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Functional Analyses of Capsid Regions in Rubella Virus Replication

by

Lok Man (John) Law ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of *Doctor of Philosophy*

Department of Cell Biology

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Abstract

Rubella virus (RV) is a small enveloped positive strand RNA virus that belongs to the family the *Togaviridae*. It is the etiological agent of rubella, a generally mild self-limiting disease that is also known as German measles. However, RV is the most teratogenic infectious agent known, and *in utero* infection during the first trimester of pregnancy, often results in severe malformations to the human fetus. Despite its medical significance, the biology of RV is not well understood. To this end, our laboratory is focused on the role of the capsid protein in virus assembly and host cell interactions. Capsid is a major structural component of rubella virions. During virus assembly, the functions of RV capsid are to package the RNA genome and to interact with the virus glycoproteins. These heterotypic binding reactions are required to coordinate nucleocapsid formation and drive virus budding respectively. In addition to its structural roles, capsid protein has been shown to modulate genome replication most likely through interactions with nonstructural proteins. In addition, capsid binds to a variety of host cell proteins and thus may be an important factor in virus-host interactions.

In the present study, the roles of two capsid regions in RV replication are investigated. First, I showed that the hydrophobic carboxyl terminus of capsid is required for membrane association of this protein. Moreover, my data indicate that this domain is required for transport of capsid to the juxtanuclear region where virus budding occurs. Secondly, I mapped a group of phosphorylated amino acid residues to the RNA binding site of capsid. Phosphorylation of serine 46 is critical for downstream phosphorylation of other amino acid residues in capsid. Dynamic phosphorylation of capsid appears to regulate the RNA binding activity of this protein and ultimately virus replication. In

summary, this work provides the basis for a mechanistic understanding of the spatial and temporal interactions of capsid during virus assembly.

Acknowledgements

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Table of abbreviations

ATP	adenosine triphosphate
BHK	baby hamster kidney
bp	base pair
°C	degrees Celsius
cDNA	complementary DNA
Ci	Curie
CIAP	calf intestinal alkaline phosphatase
CKII	casein kinase II
CTP	cytosine triphosphate
cm	centimeter
CRS	congenital rubella syndrome
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
g	gram
g	gravitational force
GST	glutathione S-transferase
HEK293T	human embryonic kidney 293T
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
IgG	immunoglobulin G
kDa	kilo Dalton
M	moles per litre
mA	mili Ampere
μg	microgram
μl	microlitre
mg	milligram
ml	milliliter
MMR	measles mumps rubella
mRNA	messenger RNA
MOI	multiplicity of infection
N/A	not applicable
ng	nanogram
NP-40	nonident P-40
ORF	open reading frame
p70S6K	70 kDa ribosomal protein S6 kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFU	plaque forming units
pH	-log[H^{+}]
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PMSF	phenylmethylsulfonyl fluoride

PP	protein phosphatase
poly (A)	poly-adenylate
PVDF	polyvinylidenelfluoride
RK-13	rabbit kidney-13
RLP	rubella like particle
RNA	ribonucleic acid
RV	rubella virus
S	Svedberg unit
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SP	signal peptide
U	units of enzyme activity
v	volume
V	Volts
VSV G protein	Vesicular Stomatitis virus glycoprotein
w	weight

Chapter 1 . Introduction

1.1 Pathology, history, and recent medical progress

Rubella virus (RV) is the etiological agent of German measles. RV infection is systemic in nature and is spread from person-to-person by respiratory aerosols. The symptoms of acute RV infection include maculopapular rash, lymphadenopathy, low-grade fever, conjunctivitis and sore throat. In most cases, these clinical features are generally mild and self-limiting (for review (Banatvala and Brown, 2004; Chantler, 2001)). In fact, it is estimated that more than half of all cases of RV infection are asymptomatic (Frey, 1994).

Natural RV infections can be complicated by the appearance of acute arthralgia or arthritis. Incidence rates for arthralgia or arthritis exceed 60% in some rubella outbreaks (Heggie and Robbins, 1969). These joint maladies occur more often in infected adult women with more severe symptoms than in men. Symptoms are usually transient, but chronic arthritis following RV infection has been observed (Chantler et al., 1982; Mitchell et al., 1993). Other rubella related complications include thrombocytopenia and encephalopathy. In very rare instances, rubella-induced encephalomyelitis can be fatal (Lau et al., 1998).

The most significant medical consequences of RV infection are the teratogenic effects, which are mainly limited to within the first trimester of pregnancy, and incidences sharply decline as pregnancy progresses. In severe cases, RV infection during this first trimester can cause premature delivery and stillbirth. However, the majority of infected fetuses survive. Over 80% of these infected fetuses develop into newborns that have severe birth defects including cataracts, cardiac defects, deafness and mental retardation. Collectively, these symptoms are known as congenital rubella syndrome (CRS) (Cooper et al., 1969).

Congenital RV infection has also been linked to several chronic diseases. As with postnatal RV infection, CRS patients often display chronic arthritis. In addition, CRS patients have a higher incidence of diabetes and thyroid disorders that appear later in life (Forrest et al., 2002). Type 1 diabetes mellitus is the most common manifestation of the delayed-onset disease. About 20% of CRS patients develop type 1 diabetes mellitus (Forrest et al., 2002; Ginsberg-Fellner et al., 1984). Although the cause of type 1 diabetes is not known, RV is the only virus known to have a direct link with the risk of accelerating the onset of type 1 diabetes (Clarke et al., 1984; Rubinstein et al., 1982). Based on epidemiological studies, previous exposure to RV among other childhood viruses has also been linked to a risk of developing multiple sclerosis. (Alter et al., 1987; Alvord et al., 1987; Compston et al., 1986; Felgenhauer et al., 1985). Moreover, CRS patients have an increased risk of developing neurological diseases such as schizophrenia (Brown et al., 2001) and, in very rare cases, a fatal neurodegenerative disorder called progressive rubella panencephalitis (Frey, 1997). Most of the transient and chronic disorders associated with RV are attributed to persistence of the virus in infected individuals or to molecular mimicry of viral proteins, which trigger autoimmune responses (Clarke et al., 1984; Ou et al., 1999).

Rubella, or German measles, was first described by two German physicians in the mid-18th century (Maton, 1815). It is primarily a childhood disease and humans are the only known host. Generally, exposure to the virus, results in the development of life-long immunity against the virus. The most obvious symptom of RV infection is the maculopapular rash that is often misdiagnosed as measles, an infectious disease that has a much higher morbidity. Due to the benign nature of RV, the disease has also been named the "3-day measles". It was not until 1941, when Dr. Norman Gregg discovered the teratogenic effects of RV, that people realized the serious medical consequence of rubella (Gregg, 1941). At that time, the placenta was thought to be impenetrable by any pathogen. The idea that RV could cross the placenta and act as a teratogenic agent was a revolutionary concept at the time. In fact, RV is the most severe infectious teratogenic agent so far identified.

RV was successfully isolated in 1962 from infected patients (Parkman et al., 1962; Weller and Neva, 1962). This was followed by the production of several live attenuated vaccines and the first RV vaccination program was implemented in the United States in 1969. After the introduction of the vaccine, the number of cases of rubella in the U.S. dropped from 57,686 in 1969 to 225 in 1991. The number of CRS cases decreased from 81 to 2 over the same period (Schluter et al., 1998).

RV vaccine is given as a part of a trivalent mixture (MMR) that also includes measles and mumps vaccine. The role of RV vaccination programs is to maintain herd immunity in order to protect susceptible women of childbearing age. In spite of the vaccination program, regular outbreaks of rubella still occur (Reef et al., 2002). In developed countries, vaccination has stopped the endemic spread of the virus (Reef et al., 2002). However, due to unwarranted fears over the MMR vaccine, based mostly on a single study that links the MMR vaccination to autism (Wakefield et al., 1998), vaccine coverage has dropped recently, especially within the United Kingdom. This drop in coverage has occurred even though most of co-authors in the study recently retracted their interpretation (Murch et al., 2004). In addition, numerous other studies have validated the safety of the vaccine (Dales et al., 2001; Kaye et al., 2001; Madsen et al., 2002; Taylor et al., 2002). As a result of the MMR concerns, public health officials have warned of a possible comeback of both measles and rubella in developed countries (Devi et al., 2002).

In developing countries, rubella remains a serious problem. Although more developing countries, especially in the Americas, have started to implement regular vaccination programs (Robertson et al., 2003), it is estimated that as many as 236,000 cases of CRS occur in developing countries during non-epidemic years (Banatvala and Brown, 2004). Furthermore, immigrants from these developing countries are the major source of RV being introduced back into industrialized countries (Sheridan et al., 2002). This situation indicates more intense effort is required to completely eradicate this vaccine-preventable disease.

1.2 *Togaviridae*: genome organization and virion structure

RV is an enveloped positive strand RNA virus in the family *Togaviridae*. The family is separated into two genera, Alphavirus and Rubivirus. RV is the sole member of the genus Rubivirus. Within the family, alphaviruses are the far better studied genus, which comprise a group of mainly arthropod-transmitted viruses. Type members of alphaviruses are Sindbis virus and Semliki Forest virus (for review, (Griffin, 2001)).

All togaviruses share a common genome organization and replication strategy. The genome is a capped RNA molecule of about 10,000-12,000 nucleotides with a poly (A) tail. The genome encodes two polycistronic open reading frames (ORFs). The 5' proximal ORF encodes the non-structural proteins required for virus replication and the 3' proximal ORF encodes the structural proteins needed for virus assembly.

Virions appear as 50-70 nm spherical particles, with an electron-dense core surrounded by a host-derived envelope (for review, (Murphy, 1980)). The diameter of the membrane enclosed nucleocapsid core is 30-40 nm, which is composed of a single molecule of RNA genome and multiple copies of capsid. On the surface of virions, the outer membrane is denoted by glycoprotein-containing fringes. In alphaviruses, both core and glycoproteins are arranged in a T=4 symmetry (Fuller and Argos, 1987; Paredes et al., 1993). Distinct from alphaviruses, the RV core has a T=3 icosahedral symmetry (Matsumoto, 1974), but the symmetry of the RV glycoproteins is not known. Figure 1.1 illustrates the schematic of a rubella virion.

The buoyant density of rubella virions in sucrose gradients is 1.18-1.19 g/ml. In comparison, the buoyant density of alphavirions is 1.20 g/ml. The electron-lucent zone between the core and host-derived membrane is wider in rubella virions compared to alphavirions, which are more compact and could account for the lighter density observed. The sedimentation coefficient of rubella virions has been reported to range between 240S and 350S (Bardeletti et al., 1975; Russell et al., 1967; Thomssen et al., 1968). The reason for this wide range of virion density is not known, but may possibly be explained by contamination of host membranous material. This heterogeneous nature of rubella virions has impeded the progress of structural studies.

1.3 Models to study RV

Despite the medical importance of RV, our understanding of the biology of this virus is relatively limited. One of the main reasons is the lack of a suitable animal model

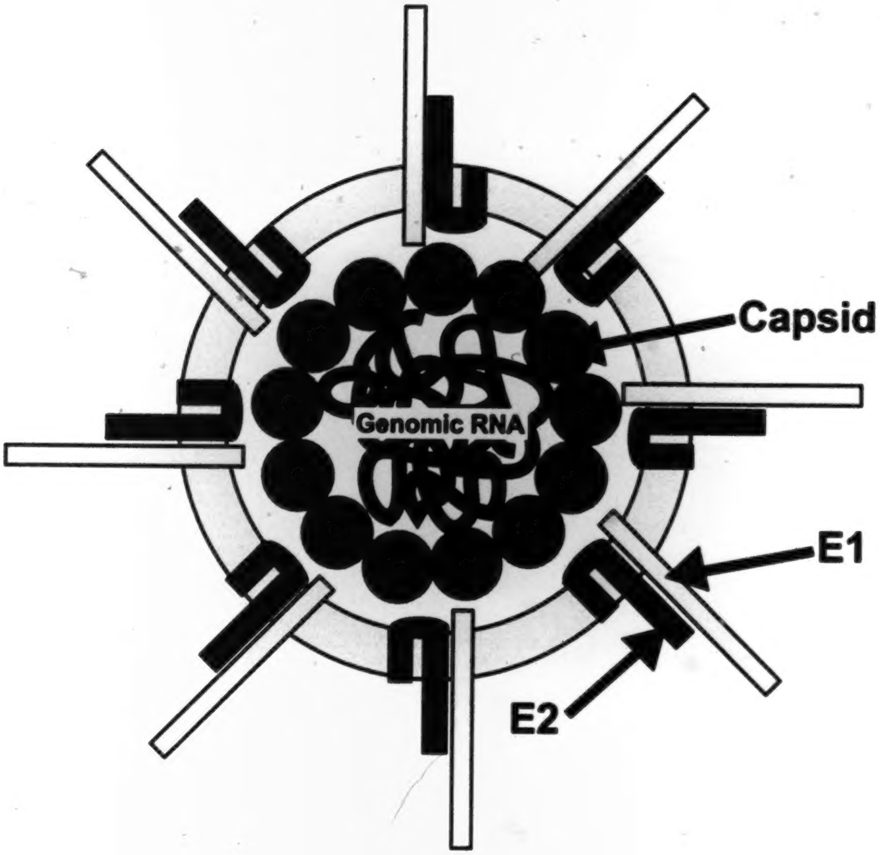


Figure 1.1. Schematic of RV. The host-derived lipid envelope contains two virus-encoded transmembrane glycoproteins (E2 and E1). The nucleocapsid of RV is composed of multiple copies of capsid and a single molecule of genomic RNA.

system to study RV. Although structurally similar to alphaviruses, RV has no arthropod vector and humans are the only known reservoir of the virus. Primates, marmosets, rats, rabbits and ferrets have been used for the study of RV pathogenesis (Horstmann, 1969; Kono et al., 1969; Rorke et al., 1968; Sato et al., 1976), but none of these animals has proven to be a reliable model. For example, although marmosets demonstrate a similar immunological response to that observed in human infections, infected animals were mostly asymptomatic and the virus did not reach the fetus (Patterson et al., 1973).

Most of the molecular biological data for RV were gathered through the use of infected cultured cells. BHK-21 (baby hamster kidney) cells and Vero (African green monkey kidney) cells are the two most utilized cell lines. Both cell lines lack a functional interferon system and allow replication of RV to relatively high titers. Although replication of RV is sensitive to interferon, persistent infections in interferon competent cells can occur (Stanwick and Hallum, 1974). The other commonly used cell line is RK-13 (rabbit kidney) cells. This cell line is particularly useful for studying the cytopathic effects of RV replication (Pugachev and Frey, 1998b; Taylor-Robinson et al., 1964).

1.4 Virus Life Cycle

For many years it was assumed that the RV life cycle is very similar to that of alphaviruses. However, several key differences between their life cycles have been noted. The membrane association of RV capsid is thought to confer several key differences in the virus assembly pathway (see section 1.7, virus assembly). This section will present a brief overview of the RV life cycle (Figure 1.2). A more detailed discussion of the specific steps will be presented in later sections.

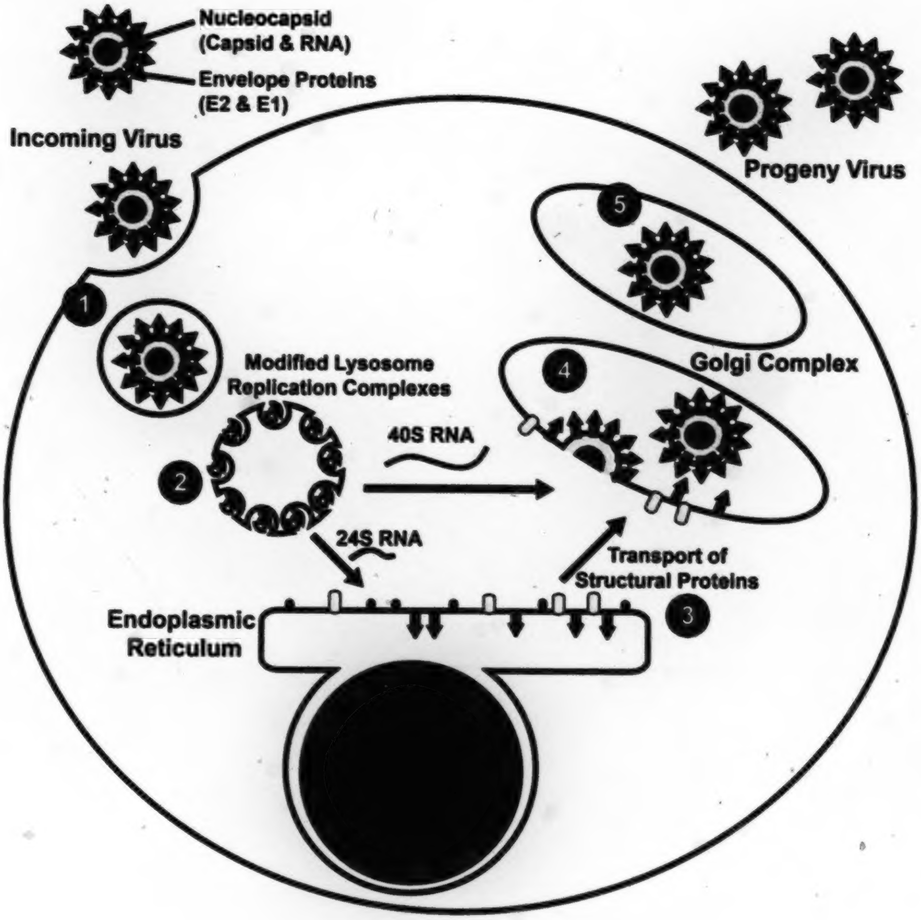

Nucleocapsid
(Capsid & RNA)
Envelope Proteins
(E2 & E1)
Incoming Virus
Progeny Virus
5
1
4
Golgi Complex
Modified Lysosome
Replication Complexes
40S RNA
2
24S RNA
Transport of
Structural Proteins
3
Endoplasmic
Reticulum

Figure 1.2. Replication cycle of RV. 1) Uptake of RV is dependent upon receptor mediated endocytosis. The low pH of the endosome/lysosome induces virus uncoating. 2) RNA replication occurs at the cytopathic vacuoles that originate from endosomes/lysosomes. Genome amplification and synthesis of the 24S subgenomic RNA also occurs at these sites. 3) Synthesis of structural proteins takes place at the ER and processing by the host cell signal peptidase separates the polyprotein into individual structural proteins. Subsequently, the structural proteins assemble and are transported to the Golgi complex. 4) Nucleocapsid assembly and virus budding occur at the Golgi complex. 5) Rubella virions undergo a series of maturation events before exocytosis.

In RV infected cells, the latent period of viral RNA and proteins synthesis is approximately 10 to 12 hours (Hemphill et al., 1988; Sedwick and Sokol, 1970; Vaheri and Vesikari, 1971). Production of progeny viruses is followed shortly after the latent period. The peak of virus titers is reached between 36 to 48 hours post-infection and is in the range of 10^6 to 10^7 plaque forming units (PFU) per milliliter. This peak virus production is 10-100 times lower than that of alphaviruses (Strauss and Strauss, 1994). Furthermore, the latent period of alphaviruses is much shorter, with their macromolecules being detected by two hours post-infection and maximal virus production occurring four to eight hours post-infection (Strauss and Strauss, 1994). The reason for these differences are not completely clear, but one possible explanation is that the high GC content (69%, the highest GC content among sequenced RNA viruses) and the unusual codon usage of the RV genome cause less efficient replication (Frey, 1994).

1.4.1 Attachment and virus entry

RV gains entry into the host cells via the receptor mediated endocytosis (Kee et al., 2004; Petruzziello et al., 1996). The host cell receptor for RV has yet to be identified. Based on the systemic nature of RV infection, the receptor is thought to be expressed ubiquitously and may in fact be a lipid. This is because treatment of cells by phospholipases A2 and C inhibits RV infection, whereas protease or glycosidase treatment has only limited effects (Mastromarino et al., 1990). In lysosomes, the low pH triggers uncoating of virions and release of the genome. While the exact mechanism of virus uncoating is poorly understood, two pH dependent changes in RV structural proteins are thought to be important for this processes: 1) The RV glycoprotein E1 on the surface of rubella virions becomes fusogenic at low pH (Katow and Sugiura, 1988); and

2) The capsid protein undergoes a structural change that results in it adopting a hydrophobic nature (Mauracher et al., 1991).

1.4.2 Replication of RV genome

Within infected cells, there are three species of RV RNA (Figure 1.3): 1) A genome-length positive sense RNA with a sedimentation coefficient of 40S; 2) A negative sense genome length RNA complimentary to the 40S genome; 3) A subgenomic RNA with a sedimentation coefficient of 24S. This RNA is translated into the structural proteins (Hemphill et al., 1988; Hovi and Vaheri, 1970b; Sedwick and Sokol, 1970). The 40S genome-length RNA is infectious when transfected into cells (Hovi and Vaheri, 1970a; Sedwick and Sokol, 1970; Wang et al., 1994). Initially, the genomic RNA functions as a mRNA that is translated into RV non-structural proteins. The non-structural proteins then form the viral replicase to transcribe the negative strand RNA, which serves as the template for genome amplification and subgenomic RNA transcription. Most of the negative strand RNA templates exist as double strand intermediates bound to either transcribing genomic or subgenomic RNAs (Hemphill et al., 1988; Sedwick and Sokol, 1970). The sub-genomic RNA is co-linear with the 3' proximal ORF and serves as the mRNA for the synthesis of structural proteins (Figure 1.4, see section 1.5.2). At a later stage of infection, the RV genomic RNA is packaged into virions and subsequently released from the cell to complete the replication cycle. The packaging signal of the genome is located between nucleotides 347 to 375 (Liu et al., 1996b). Although the subgenomic RNA is non-infectious, a small fraction of the subgenomic RNA is known to be packaged into virions (Wang et al., 1994).

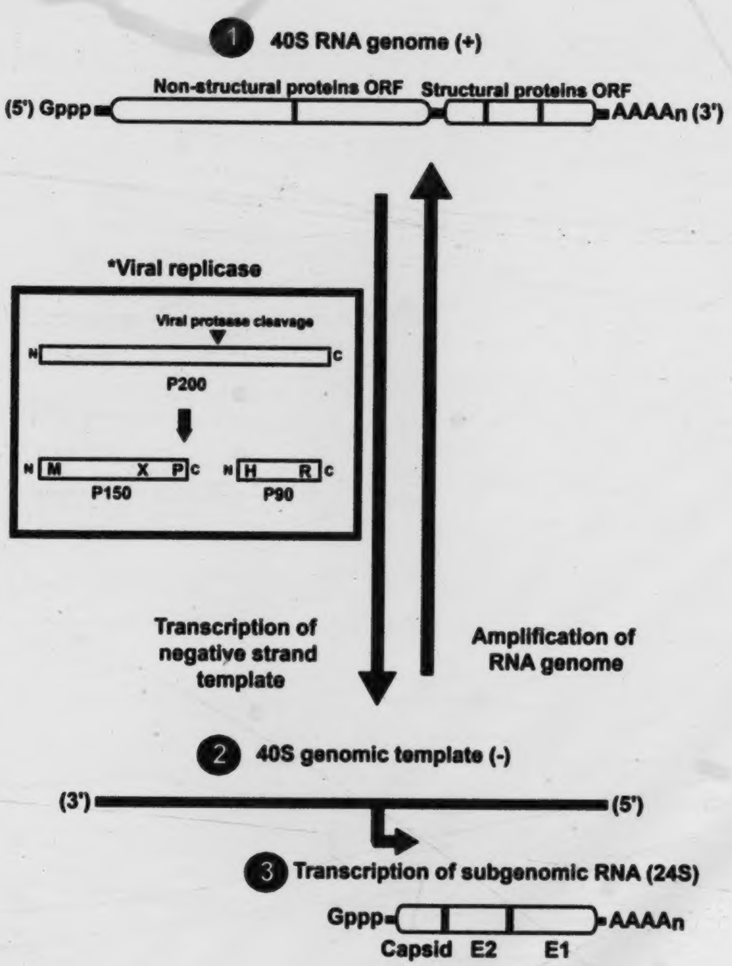

1
40S RNA genome (+)
Non-structural proteins ORF
Structural proteins ORF
(5') Gppp
AAAAn (3')
*Viral replicase
Viral protease cleavage
N
C
P200
N
M
X
P
C
P150
N
H
R
C
P90
Transcription of negative strand template
Amplification of RNA genome
2
40S genomic template (-)
(3')
(5')
3
Transcription of subgenomic RNA (24S)
Gppp
AAAAn
Capsid
E2
E1

Figure 1.3. RV replication scheme. 1) The RV genome is a 40S positive strand RNA genome that is capped and has a poly (A) tail. The genome encodes two polycistronic open reading frames (ORFs). The 5' proximal ORF encodes non-structural proteins and the 3' proximal ORF encodes structural proteins. The RNA genome first functions as a mRNA for translation of the non-structural proteins which are then processed to form the viral replicase (*). 2) The viral replicase is responsible for synthesis of the negative strand RNA template (black arrow), which is then used as a template to amplify more virus genome and to transcribe the 24S subgenomic RNA (blue arrows). 3) The subgenomic RNA functions as a mRNA for the translation of the structural proteins. The viral replicase is responsible for RV RNA synthesis. (M) methyltransferase domain; (X) X-domain; (P) protease domain; (H) helicase domain; (R) RNA-dependent RNA replicase domain.

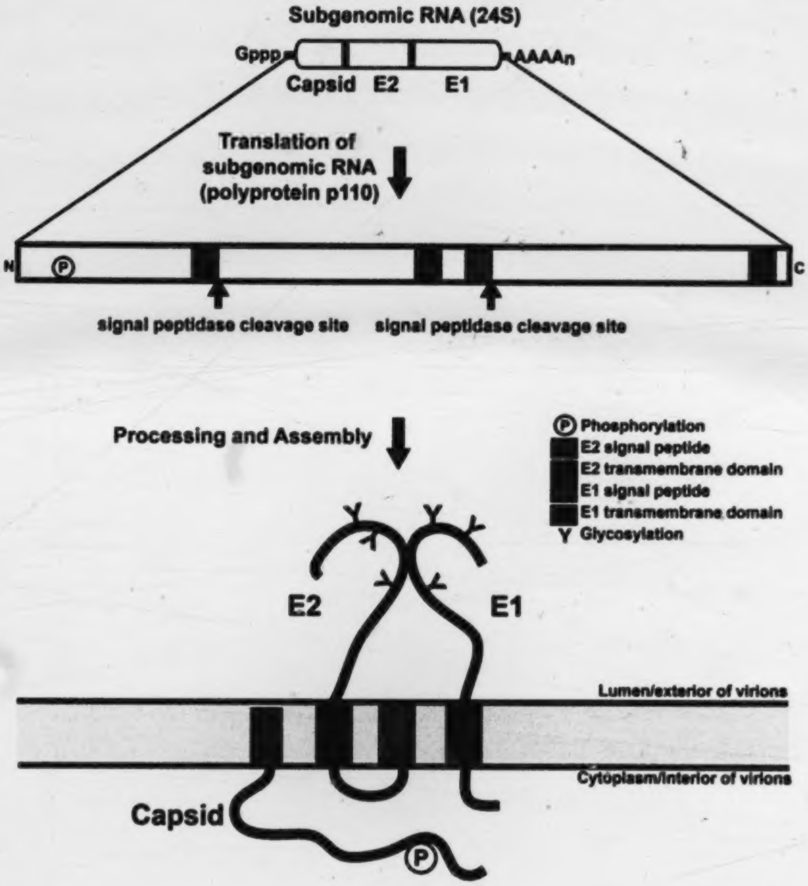

Subgenomic RNA (24S)
Gppp
AAAAn
Capsid
E2
E1
Translation of subgenomic RNA (polyprotein p110)
N
Ⓟ
C
signal peptidase cleavage site
signal peptidase cleavage site
Processing and Assembly
Ⓟ Phosphorylation
E2 signal peptide
E2 transmembrane domain
E1 signal peptide
E1 transmembrane domain
Y Glycosylation
E2
E1
Lumen/exterior of virions
Cytoplasm/interior of virions
Capsid
Ⓟ

Figure 1.4. Processing of RV structural proteins. Structural proteins are translated from the 24S subgenomic RNA as a polyprotein. The order of the polyprotein is NH_2-Capsid-E2-E1-COOH. The signal peptides of E2 and E1 initiate the translocation of glycoproteins into the lumen of the ER. Host cell signal peptidase cleaves at the carboxyl terminus of each signal peptide resulting in separation of the polyprotein into individual proteins: Capsid is a phosphoprotein located in the virion interior. E2 and E1 are membrane glycoproteins located on the surface of virions. Protein phosphorylation (P) and glycosylation (Y) are indicated. The lower part of the figure depicts the predicted membrane topology of the RV structural proteins. After processing by host cell peptidase, the bulk of capsid faces the cytoplasm (which is topologically equivalent to the interior of the virion after virus assembly). The glycoproteins face the ER/Golgi lumen (which is spatially equivalent to the exterior of the virion after virus assembly). Lateral interactions between the membrane spanning domains of E2 and E1 may be important for structural protein interactions (see text).

1.4.3 Structural protein synthesis and virus assembly

As with most enveloped viruses, RV assembly is dependent upon the host secretory pathway (see section 1.7, virus assembly). The 24S subgenomic RNA encoding the structural proteins of RV is translated at the ER (Marr et al., 1991; Oker-Blom et al., 1984). Then, structural proteins are specifically targeted to the Golgi complex, which is the primary site of virus assembly (Garbutt et al., 1999). In this respect, assembly of RV is distinct from alphaviruses, which assemble at the plasma membrane. In addition, the formation of RV nucleocapsids coincides with virus budding into the lumen of Golgi (Frey, 1994). Subsequently, the virus undergoes a structural maturation process in the Golgi complex prior to being released from cells.

1.5 RV genome and viral proteins

The complete RV genome sequences of several wild type and vaccine strains have been determined (Dominguez et al., 1990; Kakizawa et al., 2001; Pugachev et al., 1997a; Zheng et al., 1989). The RV genome is a positive strand RNA molecule that has a 7-methyl guanosine cap at the 5' end. The 3' end contains a poly (A) tract with a mean length of 53 nucleotides (Oker-Blom et al., 1984; Wang et al., 1994). The RV genome encodes two non-overlapping ORF; The 5' proximal ORF encodes non-structural proteins that are essential for viral replication and the 3' proximal ORF encodes the structural proteins, which are translated from a 24S subgenomic RNA (Figure 1.3). Using the first completely sequenced Therien strain of RV as an example, the genome, excluding the cap structure and poly (A) tail, is 9756 nucleotides in length (Dominguez et al., 1990). The 5' proximal ORF and the 3' proximal ORF contain 6385 nucleotides and

3189 nucleotides respectively (Dominguez et al., 1990). The subgenomic RNA also has a 5' cap structure and a poly (A) tail (Oker-Blom et al., 1984).

Each of two RV polycistronic ORFs is translated into a polyprotein (Marr et al., 1994; Oker-Blom et al., 1984). These two polyproteins are subsequently processed into five different proteins (two non-structural proteins, p150 and p90 (Marr et al., 1994), and three structural proteins, capsid, E2 and E1 (Clarke et al., 1987)). Processing of these polyproteins is independent and mechanistically distinct from each other. For example, processing of the non-structural proteins requires a virus encoded protease (Marr et al., 1994; Yao et al., 1998), and processing of structural proteins is dependent on host signal peptidase (Marr et al., 1991; Oker-Blom et al., 1990). Non-structural proteins are translated directly from the genomic RNA and subsequently form the viral replicase. In contrast, structural proteins are translated from the subgenomic RNA (Figures 1.3 and 1.4). Furthermore, synthesis of non-structural and structural proteins takes place at different locations. While synthesis of non-structural protein occurs in the cytosol or in association with the replication complexes (see section 1.6, RV replication), translation of structural proteins takes place at the ER.

1.5.1 Non-structural proteins

The 5' proximal ORF contains 6345 nucleotides, which encode a 200 kDa polypeptide that is cleaved to produce p150 and p90 (Bowden and Westaway, 1984; Marr et al., 1994). The protein order of the 5' ORF is NH_2-P150-P90-COOH (Marr et al., 1994) (Figure 1.3). A similar strategy is shared by alphaviruses, except alphaviruses encode for four distinct non-structural proteins (nsP1, nsP2, nsP3, nsP4) (Strauss and Strauss, 1994).

p150 is comprised of 1300 amino acid residues (Marr et al., 1994) and contains protease and methyltransferase domains (Chen et al., 1996; Koonin et al., 1992; Rozanov et al., 1992). The protease activity maps to a papain-like domain that is also conserved in alphaviruses (Chen et al., 1996). Cys1151 and His1272 make up the catalytic dyad that cleaves between Gly1300 and Gly1301 separating p200 into p150 and p90. Site-directed mutagenesis of Cys1151 abolishes the protease activity of p200 (Chen et al., 1996; Yao et al., 1998). At the amino terminus of p150 is a methyltransferase domain that is similar to those found in members of the alphavirus-like super group of positive strand viruses. The methyltransferase has been implicated in capping of virus genomes (Koonin et al., 1992; Rozanov et al., 1992). Interestingly, there is also a well-conserved X-domain in p150, which is the domain with the highest degree of similarity between RV and alphavirus proteins. In fact, X domains are conserved beyond togaviruses and extend to coronaviruses and hepatitis E virus (Gorbalenya et al., 1991). The function of this domain is not known, but it is essential for alphavirus replication (Hahn et al., 1989).

p90 is comprised of 905 amino acid residues (Marr et al., 1994) and contains both replicase and helicase motifs (Gros and Wengler, 1996; Kamer and Argos, 1984; Koonin et al., 1992). Amino acid residues 1965-1967 make up a GDD motif, a tripeptide signature of RNA-dependent RNA polymerases (Wang and Gillam, 2001). In addition, a predicted helicase domain in the amino terminus of the protein, which behaves as a RNA-stimulated nucleotide triphosphatase *in vitro*, is thought to be responsible for the unwinding of the RNA template during virus replication (Dominguez et al., 1990; Gros and Wengler, 1996; Koonin et al., 1992).

Within infected cells, p150 is localized to 30-60 nm spherules lining the inner surface of cytopathic vacuoles (Kujala et al., 1999; Lee et al., 1994; Magliano et al., 1998). These structures closely resemble the replication sites of alphaviruses that are of endosomal/lysosomal origin (Froshauer et al., 1988; Lee et al., 1994). Using both confocal and immuno-electron microscopy, p150 has been localized to the same location as the newly synthesized viral RNA, consistent with its proposed role in transcription (Kujala et al., 1999). p90, the other component of the virus replicase, presumably interacts with p150 during viral replication, but currently there are no experimental data regarding the subcellular localization of p90.

1.5.2 Structural proteins

RV virions are composed of three structural proteins: a phosphoprotein capsid and two envelope glycoproteins E2 and E1 (Oker-Blom et al., 1983). These structural proteins are translated from the 24S subgenomic RNA as a polyprotein, p110. The protein order of the subgenomic RNA is NH2-Capsid-E2-E1-COOH (Oker-Blom, 1984). Processing of p110 into the individual structural proteins requires the host protein signal peptidase, which cleaves twice at the carboxyl termini of the signal peptides of E2 and E1 respectively. As a result, the polyprotein is separated into capsid, E2 and E1 (Figure 1.4). Capsid is the major protein component of the nucleocapsid core that packages the genomic RNA (Oker-Blom et al., 1983). Glycoproteins E2 and E1 are located on the surface of the virus envelope (Hobman et al., 1993; Oker-Blom et al., 1983) and function in host cell binding and membrane fusion (Katow and Sugiura, 1988).

Processing of the RV capsid from the polyprotein is different from that of alphaviruses. For alphaviruses, capsid contains a protease domain that co-translationally catalyzes the cleavage to separate capsid from the structural polyprotein. This cleavage occurs upstream of the signal peptide for the glycoprotein E2 (Melancon and Garoff, 1987) and renders alphavirus capsids free in the cytoplasm. In contrast, RV capsid does not possess protease activity and separation from the polyprotein requires the action of host signal peptidase (Clarke et al., 1987). As a result, a hydrophobic domain of 23 amino acid residues that also functions as the signal peptide of E2, remains on the carboxyl terminus of capsid (Hobman and Gillam, 1989; Suomalainen et al., 1990). This domain has been shown to be sufficient to mediate membrane association of capsid and is thought to be important for the membrane-mediated assembly pathway of RV (see section 1.7, RV assembly) (Baron and Forsell, 1991; Suomalainen et al., 1990).

1.5.2.1 Capsid

Capsid is a 32 kDa phosphoprotein (Marr et al., 1994) that migrates as a doublet on SDS-PAGE. Moreover, two species with pI values of 8.8 and 9.5 can be separated by isoelectric focusing (Waxham and Wolinsky, 1985). The reason for capsid resolving as a doublet is not known, but phosphorylation and alternative translation initiation sites have been suggested as possible explanations (Frey, 1994). As mentioned above, RV capsid does not have protease activity and consequently the E2 signal peptide (SP) is retained at the carboxyl terminus of capsid. This results in a substantial pool of the protein becoming stably associated with membranes (Suomalainen et al., 1990). During virus assembly, one of the main functions of capsid is to interact with genomic RNA to form the nucleocapsid core. The region of capsid that binds genomic RNA has been mapped to amino acid residues 28 to 56 in the amino-terminus of the protein (Liu et al., 1996b). This region of capsid also includes two stretches of arginine residues that are important for virus infectivity (Beatch, 2004).

In addition to its role in virus assembly, capsid has recently been shown to have roles in viral replication. This new function of capsid was first noted by Tzeng *et al.* who showed that expression of the amino-terminal 31 amino acid residues of capsid protein rescues the replication of a replicon that has an in-frame deletion in the p150 gene (Tzeng and Frey, 2003). The deleted region of p150 has no known function and is not homologous to any part of the capsid protein. Consequently, the exact mechanism of complementation is unknown, but it has been suggested that the role of capsid protein in replication is to stabilize and/or target RNA for transcription (Tzeng and Frey, 2003).

Another study showing the role of capsid in RV replication was reported by Chen *et al.*, in which expression of capsid protein was shown to modulate replication of replicons and virus infectivity (Chen and Icenogle, 2004). When the levels of RNA transcripts are low, expression of capsid proteins has a dramatic effect in enhancing viral replication. In fact, the magnitude of capsid-dependent enhancement appears to be inversely proportional to the level of virus RNA transcripts. For instance, capsid does not enhance viral replication when high levels of RNA transcripts are present. Rather, expression of capsid when RNA levels are high, appears to inhibit viral replication (Chen and Icenogle, 2004). It has been proposed that virus assembly is favored at high levels of capsid and RNA, a situation where capsid would be expected to sequester RNA and subsequently inhibit RNA replication (Chen and Icenogle, 2004). Indeed, expression of a recombinant capsid that promotes formation of nucleocapsids does not enhance virus replication (Chen and Icenogle, 2004). The recent results from Frey's and Icenogle's laboratories, suggest that capsid participates in virus replication by virtue of its dynamic RNA binding activity (Chen and Icenogle, 2004; Tzeng and Frey, 2003).

Evidence that capsid has multiple functions is further illustrated by the fact that the protein exhibits multiple localizations in infected cells. In accordance with the structural role of capsid, the protein has been localized at the ER, the site of structural protein synthesis (Baron et al., 1992), and the Golgi complex, the primary site of virus assembly (Baron et al., 1992; Hobman et al., 1994a; McDonald et al., 1991). In addition to the secretory pathway, capsid has been localized to the cytoplasmic surface of mitochondria and to RV replication complexes (Beatch and Hobman, 2000; Lee et al., 1994). The latter location is consistent with its role in virus replication. Moreover, colocalization of capsid and p150 has been observed (Kujala et al., 1999). The function of capsid at mitochondria is not understood, but it is interesting to note that rubella virions contain cardiolipin, a phospholipid specifically found in the inner mitochondrial membrane (Bardeletti and Gautheron, 1976).

1.5.2.2 Glycoproteins E2 and E1

Following the separation of capsid, the polyprotein is further processed by the host signal peptidase to yield two type I membrane glycoproteins E2 and E1. These proteins heterodimerize and form the spike complex on the surface of virions (Hobman et al., 1993). The main functions of these envelope proteins are to bind host cell receptors and to mediate membrane fusion at the initial stage of virus infection (Katow and Sugiura, 1988).

E2 is comprised of 282 amino acid residues and is heterogeneously glycosylated in infected cells (Lundstrom et al., 1991; Qiu et al., 1992a). The protein contains two membrane spanning domains: a 19 amino acid transmembrane domain (E2 TM) and a 20 amino acid region that also functions as the E1 signal peptide (E1 SP) (Figure 1.4) (Baron et al., 1992; Clarke et al., 1987). Between the two hydrophobic domains, there is an arginine-rich region that constitutes the small cytoplasmic domain of E2. The arginine residues in this region are essential for virus particle assembly and have been proposed to interact with the negatively charged residues found on the capsid protein (see section 1.7, virus assembly) (Garbutt et al., 1999).

E1 is a 58 kDa glycoprotein and is also part of the spike complex on the virion surface. Because it is the immunodominant antigen containing virus neutralizing and hemagglutinin epitopes, more of E1 is thought to be exposed on the virion surface than E2 (Chaye et al., 1992). E1 also contains a hydrophobic peptide (amino acid residues 81 to 109) that is required for membrane fusion and stable binding to E2 (Yang et al., 1998). Unlike E2, E1 contains only N-linked glycosylation and a single 22 amino acid transmembrane domain (E1 TM) (Hobman et al., 1988) (Figure 1.4). Following the transmembrane domain, E1 has a 13 amino acid cytoplasmic tail that is critical for RV secretion (Garbutt et al., 1999; Hobman et al., 1994a).

1.6 RV replication

1.6.1 Assembly of the replication complex

Replication of RV takes place at distinct intracellular structures termed replication complexes or cytopathic vacuoles. These structures are morphologically similar to the replication complexes described in alphavirus infected cells (Froshauer et al., 1988; Lee et al., 1994). The cytopathic vacuoles are lined with membrane-bound spherules measuring approximately 60 nm in diameter. The spherules contain thread-like inclusions and are usually attached to the surrounding vacuoles via membranous necks. Non-structural proteins p150 and viral double-stranded RNA (see section 1.5.1, non-structural proteins) have been localized to these spherules by immunogold electron microscopy indicating that they are indeed the sites of viral replication (Kujala et al., 1999; Lee et al., 1994). Furthermore, lysosomal proteins have also been localized to the replication complexes suggesting that these structures are of endosomal/lysosomal origin (Magliano et al., 1998). Thus, the replication complexes are also known as virus-modified lysosomes.

Although both RV and alphavirus replication complexes share a number of similarities, there are several key differences between them. Within alphavirus infected cells, assembled nucleocapsids can be observed around the replication complexes (Froshauer et al., 1988). Although RV capsid has been reported to localize to the cytoplasmic face of the replication complexes, the association of assembled nucleocapsids with cytopathic vacuoles is a rare event (Lee et al., 1999). Formation of the RV replication complexes coincides with the clustering of organelles such as rough ER, the Golgi complex and mitochondria around the vacuoles (Lee et al., 1996; Lee et al., 1992; Risco et al., 2003). The clustering of mitochondria around the replication complex is thought to provide the energy source for the replication process. While the close association of these vacuoles with mitochondria is also observed in alphavirus infected cells, the rearrangement of rough ER and the Golgi complex is unique to RV replication. The reason for these differences are not known, but it may have to do with the fact that RV nucleocapsid assembly is membrane-associated whereas alphavirus assembly is not (see section 1.7, virus assembly). Thus, the close proximity of the ER, the Golgi complex and the replication complexes may be important for linking the translation of capsid to packaging of the genomic RNA at the virus budding site. The membrane association of capsid (mediated by the E2 SP) may be important for this process.

1.6.2 Regulation of virus RNA transcription

As mentioned earlier (section 1.4.2), the 40S positive strand RNA genome is first copied to a complimentary negative strand template. This provides an intermediate for the transcription of 24S subgenomic RNA and replication of the genome. Synthesis of these virus specific RNAs are tightly regulated events which, in alphaviruses, are controlled by the temporal processing of the viral non-structural proteins (for review, (Kaariainen and Ahola, 2002)). Similar to alphaviruses, temporal processing of the RV non-structural polyprotein is essential to control synthesis of the negative strand RNA template, genome amplification and transcription of subgenomic RNA (Liang and Gillam, 2000; Liang and Gillam, 2001; Wang et al., 2002). While the transcription of negative strand template requires the non-cleaved non-structural p200 polyprotein, subsequent processing into p150/p90 is important for synthesis of positive strand genomic and subgenomic RNAs. Recombinant virus encoding mutations that abolish the cleavage of p200 into p150/p90, results in accumulation of negative-strand RNA template (Liang and Gillam, 2000). Subsequently, the same group showed that processing of p200 into p150/p90 is required for synthesis of both positive strand genomic and subgenomic RNAs, indicating both non-structural proteins are important components of the viral replicase (Liang and Gillam, 2001; Wang et al., 2002).

1.6.3 Cis-acting elements and host interactions

Like other RNA viruses, the RV genome forms extensive secondary structures. Some of these structures are well conserved among alphaviruses and are essential for virus replication (Dominguez et al., 1990). At both ends of the genome, as well as between the two ORFs, there are three non-coding regions predicted to have conserved stem-loop structures. These structures are believed to be important for viral transcription (Chen and Frey, 1999; Frey, 1994; Nakhasi et al., 1994) and may act as landmarks for initiating transcription/translation, or as a platform for host cell protein binding in order to regulate genome replication. Indeed, mutations within the secondary structures at the 5' end of the genome have been shown to inhibit virus replication (Pugachev and Frey, 1998a).

Several host proteins have been implicated in assisting viral replication as a consequence of their binding to these conserved stem-loop structures (Nakhasi et al., 1991; Nakhasi et al., 1990; Nakhasi et al., 1994; Pogue et al., 1993). For example, the autoantigen La interacts with the 5' conserved region of the RV genome (Pogue et al., 1996). It is thought that tight association of La with this region enhances virus translation (Duncan and Nakhasi, 1997). Another example of a host cell protein interacting with the RV genome is the calcium binding protein calreticulin that binds to the 3' end of the RV genome (Singh et al., 1994). However, it is unclear how calreticulin affects RV replication since calreticulin is located within the ER lumen and is unlikely to be accessible to viral genomic RNA. Further work is needed in order to clarify the functions of host interactions during RV replication.

1.7 Virus assembly

Expression of cDNA encoding the RV structural proteins (section 1.5.2, structural proteins) results in formation of rubella virus-like particles (RLPs). These RLPs have been demonstrated to be immunologically and structurally similar to infectious virions (Hobman et al., 1994a; Qiu et al., 1994). Accordingly, RLPs have served as a powerful system to understand parameters affecting virus assembly.

1.7.1 Targeting of structural proteins to the virus assembly site

Processing and targeting of the RV structural proteins, particularly the glycoproteins, have been studied intensively. Structural proteins are translated from the subgenomic RNA as a polyprotein. The E2 SP and the E1 SP target the polyprotein to the rough ER allowing for processing by signal peptidase into the three structural proteins (Hobman and Gillam, 1989; Hobman et al., 1988). The structural proteins are then post-translationally modified and targeted to the Golgi complex, which is the primary virus assembly site.

Shortly after translation, E2 and E1 heterodimerize in the rough ER (Hobman et al., 1993; Waxham and Wolinsky, 1983). The transmembrane domains of E2 are important for the interaction of the glycoproteins. Replacement of the E2 transmembrane domain with an analogous transmembrane domain from a type I glycoprotein (vesicular stomatitis virus glycoprotein protein (VSV G protein)) results in failure of E2 to stably dimerize with E1 (Garbutt et al., 1999). Furthermore, targeting of the glycoproteins to the budding site is E2 dependent. Expression of E1 alone results in aggregation of protein in a smooth ER-like compartment (Baron et al., 1992; Hobman et al., 1992). In contrast, transport of E2 to the viral budding site is independent of E1 (Baron et al., 1992; Hobman et al., 1990). The dimerized glycoproteins take approximately 60-90 minutes to travel from the rough ER to the Golgi complex (Hobman et al., 1993). For an as yet unidentified reason, this is much slower than the transport rate of alphavirus glycoproteins, which takes approximately 25 minutes. During the transport, addition of N-linked glycans are required for E2 and E1 to acquire proper tertiary structures and be correctly targeted to the virus budding site (Hobman et al., 1991; Qiu et al., 1992a; Qiu et al., 1992b).

The transport of the dimerized glycoproteins is well coordinated. After synthesis and translocation into the ER, E2 quickly acquires its proper conformation and its subsequent binding to E1 is thought to provide a scaffold for E1 folding (Garbutt et al., 1999; Hobman et al., 1993). The delay in transport of the heterodimer to the Golgi complex is likely due to the slow maturation of E1 in the ER. Initially, the transmembrane and cytoplasmic domains of E1 act as an ER retention signal to prevent transport from the ER before folding is completed (Hobman et al., 1997). Upon completion of E1 folding, the ER retention signal is masked, and the E2/E1 heterocomplex exits the ER. Consistent with this idea, our laboratory showed that replacement of the E1 TM and cytoplasmic domains with the analogous domains of VSV G protein resulted in faster transport of the RV glycoproteins to the Golgi complex (Garbutt et al., 1999). This suggests that the E1 TM and cytoplasmic domains retain the glycoproteins in the ER, prolonging contact with chaperones. Once the glycoproteins reach the Golgi complex, the transmembrane domain of E2, in addition to mediating the interaction with E1, also acts as a Golgi retention signal retaining the glycoprotein heterodimers at this organelle in preparation for virus budding (Garbutt et al., 1999; Hobman et al., 1995).

The mechanism by which capsid is targeted to the Golgi complex is not well understood, but interactions with the glycoproteins are thought to be important for this process (Baron et al., 1992). In particular, E2 plays an important part in directing capsid to the Golgi complex. Co-expression of capsid with a recombinant E2 protein in which the transmembrane domain was replaced by the analogous domain of VSV G protein, resulted in mislocalized capsid (Garbutt et al., 1999). Specifically, the pool of Golgi-associated capsid was not observed within these transfected cells. In addition, changing three of the five arginines in the cytoplasmic domain of E2 to alanines had a similar effect in preventing localization of capsid to the Golgi complex (Garbutt et al., 1999). These results suggest that the transmembrane domain and/or the charged residues within the cytoplasmic domain of E2 are essential for directing capsid to the virus assembly site. However, the region of capsid that is essential for interacting with the glycoproteins has not been determined. Interestingly, there are two closely spaced clusters of aspartate and glutamate residues (residues 143-151 and 183-189) in capsid protein that may be important for electrostatically interacting with the cytoplasmic domain of E2 (Garbutt et al., 1999).

1.7.2 Virus assembly and secretion

RV budding has been reported to occur at the Golgi complex and the plasma membrane depending on the cell type and time post-infection (Bardeletti et al., 1979; Murphy, 1980; Oshiro et al., 1969). Although virus budding occurs at the plasma membrane (Murphy et al., 1968), this is not the preferred site because it typically occurs at later stages of infection. Instead, RV budding is believed to occur mainly at the Golgi complex (Edwards et al., 1969; Tuchinda et al., 1969). This is supported by molecular studies showing that the structural proteins are primarily targeted to the Golgi complex in multiples cell types (Baron et al., 1992; Hobman et al., 1990; Hobman et al., 1993). The lipid composition of secreted virions also supports the occurrence of budding at the intracellular membrane (Bardeletti and Gautheron, 1976).

One of the unique features of RV among togaviruses is that the formation of nucleocapsids coincides with virus budding. At late stages of virus infection, accumulation of glycoproteins at the Golgi complex and subsequent interaction with capsid drives virus budding, an event supported by the appearance of virions in the lumen of the Golgi complex. It has been suggested that the membrane-association of RV capsid plays a role of this unique assembly process (Suomalainen et al., 1990). In contrast, alphavirus capsids form crystalline arrays of nucleocapsids in the cytoplasm of infected cells, independently of both membranes and the budding process (Froshauer et al., 1988).

After virion formation, there is evidence that RV goes through a series of maturation events prior to secretion into the extracellular space. The first indication of this was an observation by Garbutt *et al.* that virus assembly is not necessarily followed by secretion (Garbutt et al., 1999). Replacement of the transmembrane and cytoplasmic domains of E1 with the analogous domains of VSV G protein, or the deletion of the E1 cytoplasmic domain did not block budding of virus particles into the Golgi complex, but did block secretion of virions. These results were confirmed by other reports that mutations in either the transmembrane or the cytoplasmic domains of E1 by non-conservative amino acid substitutions also abolished virus infectivity, without affecting virus assembly (Qiu et al., 2000; Yao and Gillam, 2000). Together, these results suggest that the transmembrane and cytoplasmic domains of E1 are essential for acquiring the correct folding of the spike complex on the virion surface following virus budding (Garbutt et al., 1999; Qiu et al., 2000; Yao and Gillam, 2000). Failure to achieve the proper folding may result in the retention of virions by a quality control mechanism in the Golgi complex (Moolenaar et al., 1997). Alternatively replacement of transmembrane and cytoplasmic domains of E1 may hinder the virion maturation process by preventing expose of a positive sorting signal that is required for exocytosis (Munro, 1998). The latter explanation supports a scenario in which virions go through a series of maturation steps in order to be actively selected for secretion.

A recent study by Risco *et al.* has provided further evidence that rubella virions go through stages of maturation after virus budding (Risco et al., 2003). Using freeze-substitution electron microscopy, they identified distinct stages of nucleocapsid maturation after virus budding. The nucleocapsid shell becomes more defined during maturation. The mechanism of this change remains unknown, but proteolytic cleavage of E2 SP from capsid is one possibility (Risco et al., 2003). In addition, Risco and colleagues have reported that only the "mature" form of virions are secreted. This is in agreement with the idea of Garbutt *et al.* that the process of virion maturation may involve positive selection for secretion (Garbutt et al., 1999).

1.8 Effects on host cells

Togaviruses, as well as other RNA viruses, have the ability to rearrange cellular membranes in order to facilitate efficient viral replication (Froshauer et al., 1988; Lee et al., 1992). In RV infected cells, the ER, Golgi complex and mitochondria are closely arranged around the virus replication complexes (Lee et al., 1996; Lee et al., 1992; Lee et al., 1994). This arrangement is proposed to aid the efficient transfer of virus genome from the site of replication to the site of assembly. In addition, the close association with the mitochondria may provide energy for processes such as virus replication. However, the mechanism by which these processes are coordinated is poorly understood.

Whereas organelle rearrangement is common in togavirus infected cells, the electron dense plaques (22-25 nm in thickness) found between membranes of various organelles within RV infected cells are unique (Lee et al., 1996). These plaques, also known as confronting membranes or confronting cisternae, are commonly found between outer membranes of mitochondria and rough ER, between outer membranes of adjacent mitochondria, and between adjacent ER membranes respectively (Lee et al., 1996). Since capsid forms electron dense structures (nucleocapsids) and has been localized to the cytoplasmic face of these organelles (Baron et al., 1992; Beatch and Hobman, 2000; Lee et al., 1996; Suomalainen et al., 1990), it is reasonable to assume that this protein is a component of the plaques. However, the functional significance of these structures remains speculative. Another unique feature in RV infected cells is that the mitochondria take on a club-shaped appearance and display a loss of cristae (Lee et al., 1996). These observations reflect the close link between replication and mitochondria in RV biology. Moreover, capsid may affect mitochondrial physiology through its interaction with p32, a host encoded mitochondrial matrix protein (Beatch and Hobman, 2000).

RV persistence can be easily established following infection (Kouri et al., 1974). In embryonic mesenchymal cells and retinal pigment epithelial cells, RV infection is considered to affect mainly the non-essential functions of cell, thereby allowing the survival of infected cells (Williams et al., 1993; Yoneda et al., 1986). The only noticeable effect in persistently infected cells is a slower growth rate. This effect is accentuated in primary diploid cells derived from human fetal organs (Rawls, 1968), which could explain the underdevelopment of organs in CRS fetuses. Furthermore, numerous reports have shown that RV infection affects cellular growth by inhibiting mitosis (Vaheri and Cristofalo, 1967), disrupting arrangement of actin filaments (Bowden et al., 1987) and altering growth response to epidermal growth factor (Yoneda et al., 1986).

Similar to many other viruses, apoptosis has been shown to play a role in the cytopathic response of RV in certain cell types (Duncan et al., 1999; Pugachev and Frey, 1998b). Most dying detached cells exhibit hallmark features of apoptosis such as chromatin fragmentation, membrane blebbing and DNA laddering (Duncan et al., 1999; Hofmann et al., 1999; Pugachev and Frey, 1998b). The extent of apoptosis varies among cell types and is believed to reflect the ability of RV to selectively affect the organogenesis of specific organs in CRS fetuses (Duncan et al., 1999). A caspase specific inhibitor, z-VAD-fmk has been shown to block the apoptotic effect of RV indicating that cell death occurs in a caspase-dependent manner.

The onset of RV-induced apoptosis usually coincides with the onset of viral protein synthesis (Duncan et al., 1999; Hofmann et al., 1999; Pugachev and Frey, 1998b). Moreover, ultraviolet inactivated RV does not induce apoptosis (Duncan et al., 1999; Hofmann et al., 1999; Megyeri et al., 1999) suggesting that productive infection is required for the induction of cell death. However, there are conflicting results regarding which viral proteins are involved in RV-induced apoptosis. In Vero cells, expression of RV structural proteins does not induce apoptosis (Hofmann et al., 1999) Instead, the cytopathic determinants of RV were mapped to the non-structural proteins (Pugachev et al., 1997b). These results are in contrast with a study showing that expression of capsid alone is sufficient to cause apoptosis in RK13 cells (Duncan et al., 2000). Duncan *et al.* mapped an apoptosis-inducing region to the amino-terminal 170 amino acid residues of capsid. In addition, membrane association of capsid was found to be essential for this effect (Duncan et al., 2000). The reason for the discrepancy between these studies is currently unclear, but the use of different cell types in these experiments and the levels of protein expression may account for the conflicting results.

1.9 Study of capsid

The limited coding capacity of most RNA virus genomes often requires viral proteins to be multifunctional in order to effectively transform the infected cell into a "virus factory". The RV capsid is particularly interesting in that it has a structural role (to form the virus nucleocapsid core) as well as a nonstructural role (to assist in viral replication). The localization of capsid to mitochondria also suggests that it may also affect other host cell functions. The means by which capsid performs different functions is poorly understood, but one possibility is that these functions are manifested through its interaction with multiple host cell proteins. Beatch *et al.* have shown that capsid interacts with at least four host proteins including the mitochondrial protein p32, a proapoptotic protein PAR-4, Poly (A) binding protein, and PKCζ (Beatch, 2004; Beatch and Hobman, 2000). Although the significance of these interactions remains to be determined, it demonstrates the potential of capsid to affect a diverse set of host cell functions.

1.9.1 Distinct biochemical regions of RV capsid

For the purpose of my studies, I have divided capsid into three distinct regions: I) the amino-basic region, II) the central region and III) the carboxyl-hydrophobic region (Figure 1.5). The amino-basic region contains a stretch of arginine residues, which is important for genomic RNA binding (Liu et al., 1996b). In addition, within this RNA binding site, there is a cluster of potentially phosphorylated serine/threonine residues. The central region does not share homology with any known protein domains. The function of this region has yet to be determined and possibly be required for homotypic interaction between capsids during virus assembly. The carboxyl-hydrophobic region, in addition to functioning as the E2 signal peptide, is essential in mediating membrane association of capsid (Suomalainen et al., 1990).

1.9.2 Phosphorylation of capsid

Capsid is phosphorylated prior to virus assembly (Garbutt et al., 1999; Marr et al., 1991) but the significance of this modification has not been studied further. Capsid phosphorylation is a conserved event among different strains of RV, because capsids from two different strains of RV were similarly phosphorylated in theses studies. Interestingly, a high number of predicted phosphorylated residues are clustered within the RNA binding region of capsid. Phosphorylation of other virus capsids is known to be essential for virus replication (Cartier et al., 1999; Gazina et al., 2000; Lan et al., 1999; Lu and Ou, 2002; Maroto et al., 2000; Shih et al., 1995; Wootton et al., 2002; Wu et al., 2002; Yueh and Goff, 2003). For example, phosphorylation of hepatitis B capsid protein affects a wide variety of processes such as subcellular localization of capsid, RNA encapsidation, and also viral replication (Gazina et al., 2000; Kock et al., 2003; Rabe et

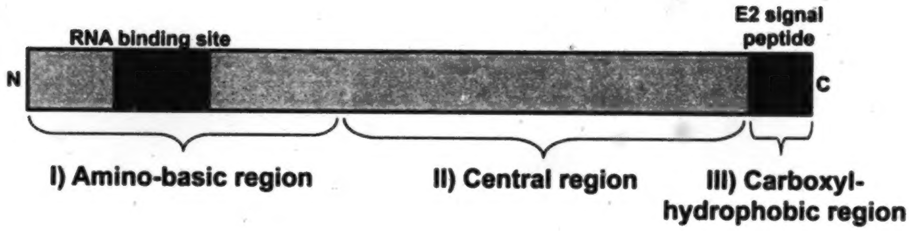

RNA binding site
E2 signal peptide
N
C
I) Amino-basic region
II) Central region
III) Carboxyl-hydrophobic region

Figure 1.5. Schematic of RV capsid protein. Capsid is composed of three distinct regions: I) The amino-terminal basic region, II) The central region and III) The carboxyl-terminal hydrophobic region. The amino-basic region includes an RNA binding site and a cluster of potentially phosphorylated amino acid residues. The central region of capsid has no known homology to other characterized protein domains. The function of this region remains to be determined. The carboxyl terminus is a hydrophobic domain of 23 amino acid residues that also functions as the E2 signal peptide. This domain is essential for capsid membrane association.

al., 2003). Although the life cycle of these viruses are distinct from RV, the capsid proteins of these viruses share a common function in that they interact with viral genomes. In addition, these examples show that capsid phosphorylation provides an additional level to regulate the functions of virus proteins. Further investigation of RV capsid phosphorylation will provide insight into how functions of this protein are regulated during viral replication.

1.10 Rationale of the project

Our lab is focused on the study of the RV capsid which is multifunctional in nature. In order to understand mechanistically how capsid performs its various functions during virus infection, the role of various regions of capsid were examined (Figure 1.5). Determining the role of the E2 signal peptide in capsid function will lead to a more thorough understanding of the unique RV assembly pathway. Furthermore, characterizing the role of capsid phosphorylation during virus infection will provide insight into the regulation of this multifunctional protein.

Chapter 2 . Materials and Methods

2.1 Reagents and Materials

Reagents and supplies listed were used as recommended by the manufacturer, unless otherwise stated.

Table 2.1 Reagents

Reagents	Source
40% Acrylamide/Bis-acrylamide solution, 29:1	Bio-Rad
Acetic acid	Fisher
Adenosine triphosphate (ATP)	Sigma
Agar	Difco
Agarose A, electrophoresis grade	Rose Scientific
Ammonium persulphate	BDH
Ampicillin	Sigma
Bacto-tryptone	Difco
Bacto-yeast extract	Difco
Baker's yeast tRNA	Roche
Bovine serum albumin (BSA)	Sigma
Bromophenol blue	BDH
Complete™ EDTA-free protease inhibitor	Roche
Coomassie Brilliant Blue	ICN
Crystal violet	Sigma
Dimethyl sulphoxide (DMSO)	Sigma
Dithiothreitol (DTT)	ICN
Dulbecco's modified Eagle's medium (DMEM)	Sigma
EPON resin (TAAB 812 resin)	Marivac
Ethanol	Commercial Alcohols
Ethylenediaminetetraacetic acid (EDTA)	Sigma
Fetal bovine serum	Atlanta Biologicals, Invitrogen, Sigma
Fibronectin	Sigma
Ficoll (type 400)	Sigma
Formaldehyde, 37% (v/v)	BDH
L-Glutamine	Gibco
Glutathione sepharose 4B	Amersham Biosciences
Glycerol	BDH
Glycine	EM Science
HEPES	Invitrogen
Hexaminecobalt chloride	Sigma
Hydrochloric acid	Fisher
Kanamycin	Sigma
Isopropanol	Fisher

Reagents	Source
Magnesium carbonate	BDH
Magnesium sulphate	Sigma
Methanol	Fisher
Minimal essential medium Eagle (MEM)	Sigma
Neutral red solution (0.33%)	Sigma
N,N,N', N',-tetramethylenediamine (TEMED)	Invitrogen
Nonidet P-40 (NP40)/IGEPAL CA-630	Sigma
Paraformaldehyde	Fisher
OptiMEM	Invitrogen
Osmium Tetroxide	Fisher
Penicillin-streptomycin solution (100 x) (cell culture grade)	Gemini Bio Products
Phenol, buffer-saturated	Invitrogen
Phosphate-free DMEM	Invitrogen
Potassium dihydrogen orthophosphate	BDH
Polyvinylpyrrolidone	Sigma
Proplyene oxide	Fisher
Protein-A-sepharose	Amersham Biosciences
Protein-G-sepharose	Amersham Biosciences
Restore™ Western Blot Stripping Buffer	Pierce
Silver nitrate	BDH
Sodium azide	Sigma
Sodium chloride	Merck
Sodium dodecyl sulphate (SDS)	Bio-Rad
Sodium fluoride	Sigma
Sodium hydroxide	BDH
Di-sodium hydrogen orthophosphate	BDH
Sodium salicylate	EM Science
Sodium orthovanadate	Sigma
Sodium thiosulphate	Sigma
Sorbitol	BDH
Sucrose	BDH
Tetrasodium pyrophosphate	Sigma
Tris base	Roche
Triton X-100	BDH
Tween 20 (polyoxyethylenesorbitan monolaureate)	Caledon
Uranyl acetate	Fisher
Vectashield mounting medium	Vector Laboratories
Xylene cyanol FF	Sigma
Yeast extract	Difco

Table 2.2 Multi-component systems

Systems	Source
FuGENE 6 Transfection Reagent	Roche
MEGAscript kit (T7)	Ambion

Systems	**Source**
mMessage mMachine High Yield Capped RNA transcription kit	Ambion
Platinum *Pfx* DNA polymerase	Invitrogen
Pwo DNA polymerase	Roche
Perfectin Transfection Reagent	Gene Therapy Systems
QIAEXII Gel Extraction kit	Qiagen
QIAquick PCR Purification kit	Qiagen
QIAGEN Plasmid Midi kit	Qiagen
Taq DNA polymerase	Invitrogen
TnT Coupled Transcription/Translation kit	Promega
Wizard Plus Minipreps DNA purification system	Promega

Table 2.3 Modifying enzymes

Enzymes	**Source**
Calf intestinal alkaline phosphatase	NEB, Roche
DNA polymerase I, large fragment (Klenow)	Invitrogen
Restriction endonucleases	NEB, Promega, Invitrogen
T4 DNA ligase	NEB, Invitrogen

Table 2.4 Radiochemicals

Radiochemicals	**Source**
Cytidine α-^{35}S triphosphate (1200 Ci/mmol)	ICN
Cytidine α-^{32}P triphosphate (3000 Ci/mmol)	ICN
Phosphorus-33 ($H_3{}^{33}$PO4) (10 mCi/ml, carrier-free)	ICN
Phosphorus-32 ($H_3{}^{32}$PO4) (5 mCi/ml, carrier-free)	ICN
^{35}S methionine (*in vitro* translation grade) (1000 Ci/mmol)	Amersham Biosciences
Pro-Mix ^{35}S methionine-cysteine (1000 Ci/mmol)	Amersham Biosciences

Table 2.5 Detection systems

Systems	**Source**
Trans-Blot Transfer Medium-nitrocellulose membrane (0.45 μm pore size)	Bio-Rad
Immobilon-P polyvinylidene fluoride (PVDF) membrane	Millipore
Rx film (for western blotting)	Fuji
Supersignal Westpico Chemiluminescent Substrate	Pierce
X-Omat AR film (for fluorography)	Kodak

Table 2.6 Molecular size standards

Markers	**Source**
1 kb DNA ladder	Invitrogen
^{14}C-labeled protein standards	Amersham
Kaleidoscope prestained protein standards	Bio-Rad
Prestained protein ladder (10-180 kDa)	Fermentas
Prestained protein marker (broad range)	NEB

2.2 Commonly used buffers

Table 2.7 Commonly used buffered solutions

Buffered Solution	**Recipe**
2 x protein sample buffer	200 mM dithiothreitol, 4% SDS, 0.2% bromophenol blue, 20% glycerol, 100mM Tris-HCl, pH 6.8
6 x DNA gel loading buffer	40% sucrose, 0.25% bromophenol blue, 0.25% xylene cyanol FF
Denhardt's solution (250 x)	0.125 mM Ficoll 400, 0.140 mM polyvinylpyrrolidone, 0.735 mM BSA
Dephosphorylation buffer	0.05 M Tris-HCl, 1mM EDTA pH 8.5
FSB	10 mM KOAc, pH 7.5, 45 mM MnCl2, 10 mM CaCl2, 10 mM KCl, 3 mM hexaminecobalt chloride, 10% glycerol
Gel-running buffer (SDS-PAGE)	250 mM glycine, 0.1% SDS and 100 mM Tris Base
Lower gel buffer	0.1% SDS, 375 mM Tris HCl, pH 8.8
NP40 lysis buffer	150 mM NaCl, 2 mM EDTA, 1% NP40, 20 mM Tris HCl, pH 7.4
PBS	137 mM NaCl, 2.7 mM KCl, 8 mM Na2HPO4, pH 7.4
PBSCM	137 mM NaCl, 8 mM Na2HPO4, 1.5 mM KH2PO4, 0.5 mM CaCl2, 1 mM MgCl2, 0.05% sodium azide, pH 7.4
Northwestern probe buffer	10 mM Tris HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 x Denhardt's solution
RIPA buffer	150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40, 50 mM Tris HCl, pH 8.0
TAE	40 mM Tris-Acetate, 1 mM EDTA, pH 8.0
Transfer Buffer	200 mM glycine, 25 mM Tris base, 20% methanol
TBST	137 mM NaCl, 2.7 mM KCl, 24 mM Tris HCl, pH 7.4, 0.05% Tween20
TE	1 mM EDTA, 10 mM Tris HCl, pH 7.5
Upper gel buffer	0.1% SDS, 250 mM Tris HCl, pH 6.8

2.3 Antibodies

2.3.1 Primary antibodies

Rabbit polyclonal anti-capsid antibody (7W7) was generated by Dr. Martin Beatch in this lab (Beatch and Hobman, 2000). Mouse monoclonal anti-capsid antibody (C1) was a kind gift from Dr. Jerry Wolinsky (University of Texas, Houston, TX). Mouse monoclonal anti-E1 antibody (B2) was a kind gift from Dr. John Safford (Abbott

Laboratories, North Chicago, IL). Rabbit polyclonal anti-calnexin (C) and rabbit polyclonal anti-GST antibodies were purchased from StressGen Biotechnology Corporation, Victoria, BC and Abcam Inc., Cambridge, MA respectively.

2.3.2 Secondary antibodies

Horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG and goat anti-rabbit IgG were purchased from Bio-Rad. Texas Red-conjugated goat anti-mouse IgG and Fluorescein Isothiocyanate (FITC)-conjugated goat anti-rabbit IgG were purchased from Jackson ImmunoResearch Laboratories.

2.4 Cell lines and viruses

2.4.1 Cell lines

COS-1, BHK, HEK 293T, RK-13, and Vero cells were obtained from the American Type Culture Collection. CHO cells constitutively expressing rubella virus E2 and E1 proteins (CHO E2E1) were previously established (Hobman et al., 1992).

2.4.2 Viruses

The M33 strain of rubella virus and the infectious cDNA clone (pBRM33) were obtained from Dr. Shirley Gillam (University of British Columbia, Vancouver, BC) (Yao and Gillam, 1999).

2.5 DNA analysis and modification

2.5.1 Isolation of plasmid DNA from *E. coli*

Plasmid DNA was prepared using WizardPlus miniprep or QIAGEN plasmid midi kits. These kits are based on a modified alkaline lysis protocol (Sambrook, 1989).

2.5.2 Restriction endonuclease digestion

Typically, reactions were performed in 20 μl volumes and carried out under the optimal conditions for the enzymes (as specified by the supplier) to completely digest 0.5 to 5 μg of DNA.

2.5.3 Polymerase chain reaction (PCR)

Pwo or *Taq* polymerases were used to amplify DNA by PCR. A typical reaction contained 50-100 ng of DNA template, 30 pmol of each forward and reverse oligonucleotide primers, 5% DMSO, 0.2 mM of dATP, dCTP, dGTP and dTTP and 5 units of polymerase. Reactions were performed for 30 cycles in a DeltaCycler II system (Ericomp) or a Robocycler Gradient 40 (Stratagene).

2.5.4 Agarose gel electrophoresis

Electrophoresis grade agarose (0.8%-2% (w/v)) was dissolved by heating in TAE and 0.375 μg ethidium bromide/ml was added prior to gel setting. DNA samples were mixed with gel loading dye and then separated at 10 V/cm by an agarose gel submerged in TAE. DNA fragments were visualized using an Ultra-violet transluminator (FisherBiotech Electrophoresis Systems) or a FluoroChem FC imaging system (Alpha Innotech Corporation).

2.5.5 Purification of DNA

The QIAquick PCR Purification Kit was used to purify PCR fragments for subsequent endonuclease digestion. Following endonuclease digestion, DNA fragments were separated by agarose gel electrophoresis, excised using a clean razor blade, and then extracted using the QIAEX II Gel Extraction Kit.

2.5.6 Dephosphorylation of 5' ends

For blunt-end ligation, calf intestinal alkaline phosphatase was used to dephosphorylate the 5' ends of vector fragments to prevent self-ligation according to manufacturer's instructions.

2.5.7 Filling-in of 5' overhangs

The Klenow fragment of DNA polymerase I was used to fill 5' overhangs of DNA fragments in order to generate blunt ends. Reactions containing 1-2 μg of DNA, 5 units of enzyme and 25 μM of dNTPs were carried out for 30 minutes at 30 °C.

2.5.8 Ligation of DNA

T4 DNA ligase was used to ligate two or more DNA fragments according to the manufacturer's instructions. The molar ratio of insert to vector was normally kept at 3 to 1 for sticky-end ligation and 6 to 1 for blunt-end ligation. Typical reactions were performed in 20 μl volumes at room temperature for either 1 hour (sticky-end) or 16 hours (blunt-end).

2.5.9 Transformation of *E. coli* DH5α

Chemical competent *E. coli* DH5α were prepared as follows. Bacteria were grown at 37 °C to an OD_{600} between 0.3 to 0.5 in 30 ml 2XYT (1.6% bacto-tryptone, 1.6% bacto-yeast extract and 0.5% NaCl (w/v) in deionized water). Cells were then collected by centrifugation (4,000 x *g* for 15 minutes) at 4 °C and washed by resuspending the bacterial pellet in 5 ml ice-cold FSB (Table 2-7). The cell suspension was centrifuged again, resuspended in 3 ml FSB plus 105 μl DMSO and incubated on ice for 15 minutes. The cells were subsequently washed twice with ice-cold FSB, divided into 200 μl aliquots, frozen in a dry ice-ethanol bath and stored at –80 °C until needed.

Transformation of chemical competent DH5α was carried out according to the method of Sambrook *et al.* (Sambrook, 1989). Briefly, frozen competent cells were thawed on ice and 5-10 μl of ligation mixture or 10-100 ng of plasmid DNA was added to 50 μl of cells. This mixture was incubated on ice for 30 minutes followed by 90 seconds of heat shock in a 42 °C water bath and 2 minutes of recovery on ice. Subsequently, 1 ml of 2XYT was added to the mixture which was then shaken at 200 rpm, 37 °C for 60 minutes. An appropriate volume of the transformation mix was plated on LB plates containing specific antibiotics to screen for transformants.

Electro-competent *E. coli* DH5α were prepared as follows. Bacteria were grown to an OD600 between 0.5 to 0.8 in 500 ml LB (1% bacto-tryptone, 0.5% bacto-yeast extract and 1% NaCl (w/v) in deionized water). Cells were collected by centrifugation (4,000 x *g* for 15 minutes) at 4 °C, washed twice with 500 ml of ice-cold sterile water and once with 10 ml of ice cold 10% glycerol (v/v). Cells were resuspended in 1 ml of ice cold 10% glycerol (v/v), divided into 50 μl aliquots, frozen in a dry ice-ethanol bath and stored at –80 °C until needed.

For electroporation, cells were thawed on ice and 0.5% to 5% of a ligation mixture was added to the electrocompetent cell mixture. The cells and DNA were then transferred to a 0.1 cm gap electroporation cuvette (Bio-Rad) and submitted to an electrical pulse according to the manufacturer's suggestion for the Bio-Rad Micropulser (Bio-Rad). Immediately after pulsing, 1 ml of 2XYT was added to the cuvette. The contents were then transferred to a microfuge tube, which was placed in a 37 °C shaking incubator for 1 hour. Approximately 10% of this mixture was plated on LB plates containing the appropriate antibiotics to screen for transformants.

2.5.10 Automated DNA sequencing

All DNA fragments amplified by PCR were sequenced to verify their authenticity and to ensure the absence of second site mutations. Plasmids or PCR products were sequenced using core facilities within the departments of Biological Sciences, Biochemistry, and Cell Biology at the University of Alberta. These facilities use a method based on fluorescently labeled didoxy terminators, which are incorporated during elongation to determine the DNA sequence.

2.6 Recombinant RV capsid plasmids

All primers used for creation of recombinant cDNA are listed in table 2.8.

2.6.1 The E2 signal peptide mutants

pCMV5-CapE2SP and **pCMV5-CapΔSP** were constructed by PCR amplification using RV 24S cDNA as template and primers that contain either *EcoR* I or *Bgl* II sites. The forward primer, capsid(F), was used in combination with the reverse primers, capsid(R) or CapΔSP(R), to produce cDNA products encoding capsid containing the E2 signal peptide (SP) (CapE2SP) and capsid without the SP (CapΔSP) respectively. The reverse primer contained the inframe stop codons. PCR products were digested with *EcoR* I and *Bgl* II and then ligated into the mammalian expression vector pCMV5 (Andersson et al., 1989).

Construction of **pCMV5-CapCD8SP** was described elsewhere (Duncan et al., 2000) and **pCMV5-CapGSP** was generated using the mega-primer and PCR overlap methods as previously described (Hobman et al., 1995; Sarkar and Sommer, 1990). The primers CapGSP(F) and CapGSP(R) were used in a PCR reaction to create a cDNA that encodes the carboxyl-terminus of capsid fused to the signal peptide sequence of vesicular stomatitis virus glycoprotein (VSV G SP). This PCR product was then used as a mega-primer in combination with the outside primers Capsid(F) or BstEII(R) in two separate PCR reactions using the RV 24S cDNA as a template. The products of these two PCR reactions were combined and PCR overlap extension was used to produce the final product which was then used to replace a 1030 base pairs fragment between *EcoR* I and *BstE* II sites of plasmid pCMV5-24S to generate pCMV5-24SGSP. Subsequently, pCMV5-24SGSP were treated with *Mlu* I and *BamH* I to delete a region encoding part of E2 and E1 proteins (nucleotide 1664 to 3321 of 24S cDNA), then treated with the Klenow fragment to fill the 5' protruding ends, and finally ligated with an oligonucleotides, XbaI stop, containing stop codons in three reading frames (Table 2.8) to produce pCMV5-CapGSP. The resulting cDNA, CapGSP, encodes a polyprotein that is composed of the capsid protein fused to the VSV G SP and followed by a truncated E2 protein (amino acid residues 1 to 36 of E2). During protein synthesis, the portion encoding the truncated E2 protein is cotranslationally cleaved from the capsid protein by the host signal peptidase to generate a recombinant capsid protein with the VSV G SP on the carboxyl-terminus.

2.6.2 Truncation mutants of capsid

pCMV5-CapN was generated with the vector-specific forward primer AV11(F) and the reverse primer Cap312(R) using pCMV5-CapE2SP (Law et al., 2001) as a template. The resulting cDNA, which encodes the first 312 nucleotides of capsid followed by a stop codon and a *BamH* I site, was digested with *EcoR* I and *BamH* I then ligated into pCMV5 (Andersson et al., 1989) that had been previously digested with *EcoR* I and *Bgl* II.

pCMV5-CapC was generated with the forward primer Cap331(F) and the reverse primer Capsid(R) using pCMV5-CapE2SP as the template. The resulting PCR product encodes a ribosome-binding site followed by a translation start site, a c-myc tag and amino acids 107 to 300 of capsid, the E2 signal peptide and a stop codon followed by a *Bgl* II site. The PCR product was digested with *EcoR* I and *Bgl* II and subcloned into pCMV5.

2.6.3 Site-directed mutagenesis of capsid

pCMV5-CapA3 was generated by site-directed mutagenesis using PCR primers encoding designated mutations. A PCR primer pair (Av11(F) and PstAAA(R)) and another primer pair (PstAAA(F) and Capsid (R)) were used respectively to generate two PCR fragments using pCMV5-CapE2SP as template. These two PCR fragments encoding nucleotides 1 to 156 and 136 to 508 of capsid coding sequences respectively. Primers PstAAA(R) and PstAAA(F) are partially complement to each other. The complement region contains a engineered silence mutation to generate a *Pst* I site between nucleotides 140 and 146 of capsid. Therefore, these two overlapping PCR fragments were ligated at the *Pst* I site and subsequently replaced the *EcoR* I and *Sal* I fragment (nucleotides 1 to 508) of pCMV5-CapE2SP. The resulting plasmid (pCMV5-CapA3) encodes a RV capsid with mutations of threonine 47, serine 48 and serine 49 to alanines (Table 2.8).

pCMV5-CapA5 was generated using a mega primer CapRNA5(F), a reverse primer CapSal(R) and pCMV5 CapE2SP as the template. The resulting cDNA was digested with *Not* I and *Sal* I and used to replace a 434-bp fragment of pCMV5-CapE2SP. Similar to pCMV5-CapA3, pCMV5-CapA5 also has a silent mutation that introduces a *Pst* I site at nucleotides 140 to 146 of capsid.

pCMV5 CapA4-1 and **CapA4-2** were generated by swapping the *Not* I and *Pst* I fragment (nucleotide 140 to 165 of capsid) from pCMV5-CapA3 with the analogous fragment from pCMV5-CapA5. Both CapA4-1 and CapA4-2 contain 4 alanine substitutions within the RNA binding region of capsid (Table 4.1). In CapA4-1, serines 45, 46, 48, 52 were replaced with alanines. In CapA4-2, threonine 47 and serines 48, 52, 58 were replaced with alanines.

pCMV5-CapS34A was generated using the primer pair Av11(F) and S34A(R) and **pCMV5 T8A** was generated using the primer pair T8A(F) and PstAAA(R). Both PCRs used pCMV5-CapE2SP as template. The resulting cDNAs were digested with *EcoR* I and *Not* I and used to replace the corresponding 164-bp fragment of pCMV5-CapE2SP.

pCMV5-CapE2SP(noSalI) was generated by digesting pCMV5-CapE2SP with *Hind* III and *Xba* I, then treating with Klenow fragment to fill the 5' protruding ends followed and ligation to re-circularize the plasmid. As a result, a 24 bp fragment between *Hind* III and *Xba* I in the multiple cloning site of pCMV5-CapE2SP is deleted. Another *Xba* I site resulting from religation is created at the multiple cloning sites downstream from the capsid coding sequence.

All other site-directed mutants of capsid (**pCMV5-CapP5, CapP4, CapP3, CapS45/46A, CapS52/56A, CapS45A, CapS46A, CapP6D, CapP5D, CapP3D, CapP3E, CapS46N, CapS46D, CapS46E, CapT47A, CapT47E**) were generated using pCMV5-CapE2SP(noSalI) as the template. Forward mutagenic primers included nucleotides 109 to 168 of capsid, except primers S52/56A(F) and P6D(F), which included nucleotides109 to 179. Each forward primer specifies serine/threonine-to-alanine, asparagine, aspartate or glutamate changes in the capsid (Table 2.8). These forward primers were utilized in combination with the reverse primer CapSalI(R). Resulting PCR products were digested with *Not* I and *Sal* I and used to replace the 401-bp fragment of pCMV5-CapE2SP.

2.6.4 GST-Capsid fusion constructs

Construction of **pEBG-Cap** were described elsewhere (Law et al., 2003). **pEBG-CapS45A, CapS46A** and **CapT47A** were amplified by PCR with primers CGST(F) and AV10Cla(R) using pCMV5-CapS45A, CapS46A or CapT47A respectively as the template, PCR products were digested with *BamH* I and then ligated in-frame to the 3' end of the GST cassette in the mammalian expression vector pEBG (Mizushima and Nagata, 1990). Transcription of the GST-fusion protein is driven by the EF-1α promoter.

Table 2.8 Primers

Primer Name	Sequence (5'to 3')	Underlined Sequence	Sequence in Bold
Capsid(F)	CGCGAATTCATGGCTTCCACTACCC	*EcoR* I	N/A
Capsid(R)	GGTCAGATCTCTAGGCGCGCGCGGTGC	*Bgl* II	N/A
CapΔSP(R)	ACTGAGATCTAGCGGATGCGCCAAGGATG	*Bgl* II	N/A
CapGSP(F)	CCATCCTTGCGCATCCGCATGAAGTGCCT TTTGTACTTAG	N/A	N/A
CapGSP(R)	ATATCAGCGCGGGGCTGGAGCCCGCAATT CACCCCAATGAATAA	N/A	N/A
BstEII(R)	CCGACGCGCAAGGTGC	N/A	N/A
Cap312(R)	GGGGATCCTA**TTGCATACGCGGGGGTTG**	Stop codon	Annealing to cDNA coding for capsid
Cap331(F)	CGGAATCCGCCACCATGGAGCAAAAGCTC ATTTCTGAAGAGGACTT**GCCGCGTATGC** **AAACCGG**	Ribosome binding site	Annealing to cDNA coding for capsid
PstAAA(F)	AGCGCTGCAGGAGATGAC**GCCGGCC**GTG AC	*Pst* I	Mutated to encode for alanines

PstAAA(R)	**GGC**GTCATCTCCTGCA**GC**GCTGGAGTCGCG	*Pst* I	Mutated to encode for alanines
CapRNA5(F)	CCGCGGCCGCCGCGACAGCGCGAC**GCCGC**AACT**GCA**GGAGATGAC**GCC**GGCCGTGAC**GCC**GGAGGGCCCCGC	*Not* I	Mutated to encode for alanines
CapSalI(R)	GTAAAATGCAGGTCGACG	*Sal* I	N/A
S34A(R)	GCGGCCGCGGCCGGCG**TGC**CTGCG	*Not* I	Mutated to encode for alanine
T8A(F)	GAATTCGGAGAGCCCCAGGGTGCCCGAATGGCTTCCACTACCCCCATC**GCC**ATGGAG	*EcoR* I	Mutated to encode for alanine
P5A(F)	CCGCGGCCGCCGCGACAGCGCGAC**GCCGCAG**CTGCAGGAGATGACGCCGGCCGTGACTCC	*Pst* I	Mutated to encode for alanines
P4A(F)	CCGCGGCCGCCGCGACAGCGCGAC**GCCGCAG**CTGCAGGAGATGACTCCGGCCGTGACTCC	*Pst* I	Mutated to encode for alanines
P3A(F)	CCGCGGCCGCCGCGACAGCGCGAC**GCCGCAGCC**TCCGGAGATGACTCCGGCCGTGACTCC	*Not* I	Mutated to encode for alanines
S45/46A(F)	CCGCGGCCGCCGCGACAGCGCGAC**GCCGC**AACCTCCGGAGATGACTCCGGCCGTGACTCC	*Not* I	Mutated to encode for alanines
S52/56A(F)	CC**GCGGCCGC**CGCGACAGCGCGACTCCAGCACCTCCGGAGATGAC**GCC**GGCCGTGAC**GCC**GGAGGGCCCCGC	*Not* I	Mutated to encode for alanines
S45A(F)	CCGCGGCCGCCGCGACAGCGCGAC**GCCA**GCACCTCCGGAGATGACTCCGGCCGTGACTCC	*Not* I	Mutated to encode for alanine
S46A(F)	CCGCGGCCGCCGCGACAGCGCGACTCC**GC**AACCTCCGGAGATGACTCCGGCCGTGACTCC	*Not* I	Mutated to encode for alanine
P6D(F)	CCGCGGCCGCCGCGACAGCGCGAC**GACGACGACGAC**GGAGATGAC**GAC**GGCCGTGAC**GAC**GGAGGGCCCCGC	*Not* I	Mutated to encode for aspartates
P5D(F)	CCGCGGCCGCCGCGACAGCGCGAC**GACGACGACGAC**GGAGATGAC**GAC**GGCCGTGACTCC	*Not* I	Mutated to encode for aspartates
P3D(F)	CCGCGGCCGCCGCGACAGCGCGAC**GACGACGAC**TCCGGAGATGACTCCGGCCGTGACTCC	*Not* I	Mutated to encode for aspartates
P3E(F)	CCGCGGCCGCCGCGACAGCGCGACTCC**GAAGAAGAA**GGAGATGACTCCGGCCGTGACTCC	*Not* I	Mutated to encode for glutamates

S46N(F)	CCGCGGCCGCCGCGACAGCGCGACTCCAACACCTCCGGAGATGACTCCGGCCGTGACTCC	*Not* I	Mutated to encode for asparagine
S46D(F)	CCGCGGCCGCCGCGACAGCGCGACTCCGACACCTCCGGAGATGACTCCGGCCGTGACTCC	*Not* I	Mutated to encode for aspartate
S46E(F)	CCGCGGCCGCCGCGACAGCGCGACTCCGAAACCTCCGGAGATGACTCCGGCCGTGACTCC	*Not* I	Mutated to encode for glutamate
T47A(F)	CCGCGGCCGCCGCGACAGCGCGACTCCAGCGCCTCCGGAGATGACTCCGGCCGTGACTCC	*Not* I	Mutated to encode for alanine
T47E(F)	CCGCGGCCGCCGCGACAGCGCGACTCCAGCGAATCCGGAGATGACTCCGGCCGTGACTCC	*Not* I	Mutated to encode for glutamate
CGST(F)	AGATCTGGATCC**ATGGCTTCCACTACCCCCATC**	*BamH* I	Annealing to cDNA coding for capsid
Av11(F)	TACGGTGGGAGGTCTATATAGC	N/A	N/A
Av10cla(R)	TTGATCATCGATGGGCACTGGAGTGGCAAC	*Cla* I	N/A
T7-Av11(F)	CGAGATATCAGATCTTAATACGACTCACTATAGGGCGGTGGGAGGTCTATATAGC	N/A	N/A
Av10(R)	CAAAGGCCAGGAGAGGCAC	N/A	N/A
XbaI stop	(P)CTAGTCTAGACTAG	*Xba* I	Stop codon

2.6.5 Infectious RV cDNA clones

cDNA fragments encoding the desired capsid mutants were subcloned into a plasmid encoding the entire rubella M33 strain genome (pBRM33) by replacing the *Not* I and *Sph* I fragment (nucleotides 6622 and 7242) within the capsid-coding region (Yao and Gillam, 1999).

2.7 Protein gel electrophoresis and protein detection

2.7.1 Sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Protein samples were electrophoresed through 10% or 15% acrylamide gels. Briefly, protein samples were mixed with 1 volume of 2 x protein sample buffer (Table

2.7) and denatured by boiling for 5 minutes. The samples were then separated by discontinuous gel electrophoresis (4% stacking gel above a 10% or 15% resolving gel) in gel running buffer at 100-150 V using either the Bio-Rad mini-protean II or the Bio-Rad mini-protean III system. After electrophoresis, the gels were processed for western blotting, fluorography or silver staining as described below.

2.7.2 Western blotting

Following SDS-PAGE, proteins in the gels were transferred to PVDF membranes for immunoblotting. PVDF membranes were first wetted in methanol and then, together with gels, equilibrated in transfer buffer (Table 2.2) for 15 minutes. Protein transfer was carried out using a Mini Trans-Blot Electrophoretic Transfer cell apparatus (Bio-Rad) at constant current (260 mA) for 1 hour at 4 °C. Once the transfer was completed, the PVDF membranes were washed once with TBST (Table 2.2) and subsequently blocked with 4% skim milk in TBST for 15 minutes at room temperature. After blocking, the membranes were incubated with the appropriate primary antibody diluted in TBST with 4% skim milk for 1 hour, followed by washing three times in TBST for 5 minutes. Membranes were then incubated with Horseradish Peroxidase (HRP)-conjugated secondary antibodies for 30 minutes at room temperature. In most cases, commercially available secondary antibodies were diluted (1:3000) with TBST with 4% skim milk. Next, the membranes were washed three times in TBST for 5 minutes, followed by incubation with Supersignal Westpico Chemiluminescent Substrate as prescribed for enhanced chemiluminescence (ECL) by the manufacturer. The signals were detected using Fuji Rx film or the FluroChem FC imaging system (Alpha Innotech Corporation).

2.7.3 Fluorography

To visualize radio-labeled proteins, samples were first resolved by SDS-PAGE and the gels were fixed in 25% (v/v) isopropanol and 10% (v/v) acetic acid for 30 minutes followed by a 30 minutes incubation in 100 mM sodium salycilate containing 0.01% (v/v) β-mercaptoethanol. The gels were rinsed with deionized water prior to incubating in the sodium salycilate solution. Finally, gels were dried and exposed to Kodak XAR film at -80 °C or Storm 840 phosphorimager screen (Molecular Dynamics).

In some cases after transferring radio–labeled proteins to PVDF, membranes were air-dried and exposed to Kodak XAR film at –80 °C or a phosphorimager screen. Protein bands were quantitated by densitometry or by ImageQuant 1.2 software (Molecular Dynamics).

2.7.4 Silver staining

Following SDS-PAGE, gels were fixed in 50% (v/v) methanol and 10% (v/v) acetic acid for 30 minutes at room temperature, sensitized in 0.02% (v/v) sodium thiosulfate for 1 minute at room temperature and then treated with 0.1% (w/v) silver nitrate for 25 minutes at 4 °C. The gels were developed in 2% (w/v) anhydrous sodium carbonate and 0.02% (v/v) formaldehyde for a maximum of 10 minutes at room temperature. Development was stopped by immersing the gels in 1.4% (w/v) EDTA sodium salt solution.

2.8 Culture and transfection of mammalian cell lines

2.8.1 Mammalian cell culture

COS-1, BHK-21, HEK 293T and Vero cells were cultured in DMEM (high glucose) containing 10% fetal bovine serum, 2 mM glutamine, 1 mM HEPES, penicillin

(100 U/ml) and streptomycin (100 U/ml). RK-13 cells were cultured in MEM containing 10% fetal bovine serum, 2 mM glutamine, 1 mM HEPES, penicillin (100 U/ml) and streptomycin (100 U/ml). CHO-E2E1 cells were cultured in α-MEM containing 10% dialyzed fetal bovine serum, 2 mM glutamine, 1 mM HEPES, penicillin (100 U/ml) and streptomycin (100 U/ml). All cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2.

2.8.2 Transient transfection of cell cultures

COS-1 and HEK 293T were transiently transfected with indicated amounts of plasmid DNA using either Fugene 6 or Perfectin transfection reagents exactly as described by the manufacturers. Transfected cells were processed for experimental analysis 24-48 hours post-transfection as indicated.

2.9 Immunoprecipitation and radioimmunoprecipitation

2.9.1 Immunoprecipitation

Cells were rinsed three times with ice cold PBS, then lysed with RIPA buffer (Table 2.7) (0.5 to 1 ml per 35 mm dish) for 10 minutes on ice. Lysates were then precleared by centrifugation at 14,000 x g for 10 minutes at 4 °C to remove nuclear and insoluble material. The resulting supernatants were immunoprecipitated from the supernatant using specified antibodies for 1 hour with rotation at 4 °C followed by the addition of protein-A-sepharose beads (for rabbit antibodies) or protein-G-sepharose beads (for mouse monoclonal antibodies). Immunocomplexes were washed three times with RIPA buffer and then eluted from beads by boiling in 2 x gel loading buffer (Table 2.2).

2.9.2 Metabolic Labeling and radioimmunoprecipitation

2.9.2.1 ^{35}S-metabolic labeling of proteins

Transfected COS-1 cells were cultured for 48 hours prior to radiolabeling. Cells were washed once with sterile warm PBS followed by incubation in methionine/cysteine-free DMEM media for 30 minutes to deplete the intracellular pool of methionine and cysteine. Cells were then incubated with 100 μCi of ^{35}S-ProMix per ml of medium for 0.5 to 4 hours. After labeling, cells were processed for immunoprecipitation, SDS-PAGE and fluorography.

2.9.2.2 ^{32}P/^{33}P-labeling of proteins

Where indicated, transfected COS-1 or RV infected-Vero cells were radiolabeled with phosphorous-32 ($H_3{}^{32}PO_4$) or phosphorous-33 ($H_3{}^{33}PO_4$). Prior to labeling, cells in 35 mm dishes were washed in PBS once, incubated in phosphate-free DMEM for at least 30 minutes, then labeled in phosphate-free DMEM containing 100 μCi radioactive phosphate for 4 to 16 hours. Following labeling, cells were processed for immunoprecipitation, SDS-PAGE and fluorography. During radioimmunoprecipitation, all solutions contained phosphatase inhibitors (1 mM sodium orthovanadate, 50 mM sodium fluoride, 5 mM tetrasodium pyrophosphate).

2.9.3 GST pulldown

COS-1 and HEK 293T cells (6 x 10^5/ 60 mm dish) were transfected with plasmid DNA encoding GST-capsid fusion proteins. Two days post-transfection, cell lysates were processed similarly to immunoprecipitation procedures (Section 2.9.1), except 1% NP40 (Table 2.2) was used in the lysis buffer to preserve the integrity of protein complexes. To pulldown GST-capsid protein complexes, lysates were rotated with glutathione agarose beads for 1 hour at 4 °C. Beads containing protein complexes were washed 3 times with PBS containing 0.1% Trition X-100. Proteins were eluted from beads by boiling in 2 x protein sample buffer for 5 minutes. Samples were subjected to SDS-PAGE, fluorography or silver staining.

2.10 Microscopy

2.10.1 Immunofluorescence microscopy

COS-1 or Vero cells were cultured on fibronectin-coated coverslips for 24 hours post-transfection or infection. Coverslips were washed 3 times with PBSCM for 3 minutes each, followed by either methanol or paraformaldehyde fixation. For methanol fixation, cells were treated with absolute methanol for 6 minutes at –20 °C, followed by PBSCM washes to rehydrate the samples. This treatment both fixed and permeabilized cells. For paraformaldehyde fixation, the cells were incubated in 3% paraformaldehyde solution for 20 minutes, followed by quenching with 50 mM ammonium chloride. After fixation, coverslips were washed twice with PBSCM and then membranes were permeabilized by treating with 0.1% Triton X-100 for 10 minutes. After fixation and permeabilization, samples were blocked in PBSCM containing 1% BSA for 15 minutes, and then incubated sequentially with primary and secondary antibodies diluted in blocking solution. Following antibody treatment, cells were washed 3 times with PBSCM containing 0.1% BSA. After the post-secondary antibody washes, coverslips were mounted on slides using Vectashield mounting medium. Samples were examined using a Zeiss 510 confocal microscope. Images from optical sections (0.5 μM) were processed using Adobe photoshop 7.0.

2.10.2 Electron microscopy

CHO cells stably expressing E2E1 were transfected with various capsid constructs and, 48 hours post-transfection, were fixed with 2.5% glutaldehyde in 0.1 M cacodylate buffer (pH 7.4). Fixed samples were then pelleted at 2,400 x g for 4 minutes, embedded in Epon and then processed for routine morphologic examination as described (Garbutt et al., 1999). Briefly, pellets were washed 3 times with 0.1 M cacodylate buffer, followed by post-fixation on ice with 1% osmium tetroxide in 0.1 M phosphate buffer for 45 minutes. Samples were then dehydrated by sequential washes in 60%, 80%, 95% and 100% ethanol. Pellets were then infiltrated with a 1:1 mixture of propylene oxide and EPON embedding medium for 1 hour followed by incubation in a fresh mixture of resin for 4 hours. The resin was polymerized at 60 °C for 48 hours. Thin sections (70 nm) were collected on 300 mesh copper grids (EM Science) and then stained with uranyl acetate and lead citrate. The samples were then examined at 80 kV on an EM 410 electron microscope (Philips).

2.11 Virology techniques

2.11.1 Infection of Vero and RK-13 cells with RV

Virus stocks were diluted with cell culture media and overlaid onto PBS-washed cell monolayers (approximately 1 ml /35 mm dish). For a minimum of 4 hours at 35 °C, the virus inoculum was replaced with normal growth media and the cells were cultured at 35 °C until processing for experimental analysis.

2.11.2 Electroporation of BHK cells

Plasmids encoding full-length RV cDNA clones were linearized with *Hind* III and used as templates for transcription of capped RNAs (mMessage mMachine kit, Ambion).

RNAs were quantitated by gel electrophoresis and spectroscopy at 260 nm before introduction into BHK cells by electroporation. Briefly, sub-confluent BHK cells were trypsinized and then resuspended in PBS (1×10^7 cells/ml). Wild type and mutant viral RNAs (10 μg each) were added to 0.5 ml aliquots of BHK cell suspensions. The cell/RNA mixtures were transferred to 0.2mm gap electroporation cuvettes and incubated on ice for 10 minutes. Cells were electroporated at a 500 V, 100 μF pulse in a Electro Cell Manipulator ECM600 (BTX Electronic Genetics). Immediately after the pulse, 1.0 ml of culture media was added to each cuvette. Cells were further diluted in 11.0 ml of culture media and distributed into six 35 mm culture dishes. Virus-containing culture media collected on consecutive days, was clarified by centrifugation at 7,000 x *g* for 10 minutes before immediate use or storage at –80 °C

2.11.3 Plaque assay and determination of cytopathic effects

RK13 cells (2×10^5 cells) were infected with virus stocks in 35 mm dishes for 2 hours, washed and allowed to recover in culture media for one hour. Cells were then overlaid with warm 0.5% agarose in culture medium and then incubated at 35 °C in 5% CO_2 atmosphere for six days. Viral plaques were visualized by staining with 0.013% (w/v) neutral red solution (Sigma) for 3 hours.

Cytopathic effect was scored by examination of infected cultures by light microscopy following crystal violet staining. For staining, cells were washed with PBS and then fixed and stained with 0.05% crystal violet in 17% methanol for two hours at room temperature. Excess stain was removed by washing cells with distilled water. The stained cells were examined with a Zeiss Axioskop 2 microscope and images were captured using a Spot™ camera (Diagnostics Instruments).

2.12 Analysis of RV capsid

2.12.1 Rubella virus-like particle (RLP) and virus secretion assays

Media from transfected COS cells or infected Vero cells were pre-cleared by centrifugation at 14,000 x *g* for 10 minutes at 4 °C. The supernatants were then subjected to ultracentrifugation at 100,000 x *g* for 1 hour at 4 °C to pellet RLPs or virions. The resulting pellets was resuspended in 2 x protein sample buffer and subjected to SDS-PAGE and immunoblotting using anti-capsid antibodies (7W7).

Alternatively, media from infected cells were immunoprecipitated with monoclonal mouse anti-E1 antibody (B2) for 1 hour at 4 °C. The immunocomplexes were then collected by protein G-sepharose as described (Section 2.9.1), except that washes were done with PBS instead of lysis buffer to preserve the integrity of the virions. To test for the presence of secreted virions, samples were probed for capsid by immunoblotting.

2.12.2 *In vitro* RNA binding assay

Capsid proteins were purified from rubella virions, transfected COS-1 or infected cells by immunoprecipitation as described (Section 2.9.1). Virions were isolated from the pre-cleared culture media of infected Vero cells by centrifugation at 100,000 x g for 60 minutes at 4 °C. Where indicated, some capsid preparations were dephosphorylated prior to RNA binding experiments. Immunoprecipitated capsids were subjected to a final wash in dephosphorylation buffer (Table 2.7) and then incubated 12-15 hours at 37 °C in dephosphorylation buffer containing 100 units of calf intestine alkaline phosphatase. Protein samples were then separated by SDS-PAGE and transferred to 0.45 μm pore-size nitrocellulose membranes (Bio-Rad). Membranes were washed in northwestern probe buffer (Table 2.7) for 10 minutes at room temperature followed by blocking for 1 hour in probe buffer containing 250 μg/ml baker's yeast tRNA (Roche).

The RNA probe used for northwestern blots corresponded to nucleotides 1-4211 of the M33 genome sequence, a region that contains the RNA packaging signal (Liu et al., 1996b). An *EcoR* I and *EcoR* V cDNA fragment from pBRM33 encompassing this region was ligated into the *EcoR* I and *Sma* I sites of pBluescript SK+ (Stratagene). The resulting plasmid was linearized with *BamH* I and used as a template to generate non-capped RNA using the MEGAscript kit (Ambion). Transcription reactions (20 μl) contained one microliter of $[^{35}S]\alpha$-CTP (10 μCi, 1200 Ci/mmol) or $[^{32}P]\alpha$-CTP (10 μCi, 3000 Ci/mmol). Membranes were hybridized with radio-labeled RV RNA for one hour at room temperature in probe buffer. After hybridization, membranes were washed in probe buffer three times at room temperature before exposure to a phosphorimager screen.

2.12.3 Membrane co-pelleting assay

Capsid proteins were tested for the ability to stably associate with membranes using a previously described membrane co-pelleting assay (Suomalainen et al., 1990). Briefly, ^{35}S-labeled capsid proteins were synthesized *in vitro* using a coupled transcription/translation system, TnT (Promega) either in the presence or absence of dog pancreatic microsomes (from Dr. Chris Nicchita, Duke University, North Carolina). Templates for the TnT reaction were generated by PCR using the vector specific primers T7-Av11(F) and AV10(R) (Table 2.8). The resulting cDNA encodes full length capsid sequence preceded by a T7 promoter, which is required for the TnT reaction.

Ten percent of each reaction mixture (5 μl) was extracted at room temperature with 50 mM sodium carbonate (50 μl) for 30 minutes, followed by centrifugation in a

Beckman Airfuge set at 25 lb/in^2 through a sucrose cushion containing 0.2 M sucrose, 30 mM HEPES (pH 11.5), 150 mM KoAc, 2.5 mM magnesium acetate and 1 mM DTT. The supernatants and the pelleted membrane fractions were subjected to SDS-PAGE and fluorography. Under these conditions, only capsids that are stably associated with microsomes are recovered in the pellet fractions.

2.12.4 Identification of potentially phosphorylated residues in capsid

The capsid amino acid sequence was subjected to analyses using three different algorithms that predict potentially phosphorylated residues: 1) Prosite (http://au.expasy.org/tools/scanprosite/); 2) NetPhos 2.0 (http://www.cbs.dtu.dk/services/NetPhos/); and 3) Scansite (http://scansite.mit.edu/). All parameters were set at the default setting during the analysis. Most of the predicted phosphorylated residues are clustered within the RNA binding region of capsid (Figure 4.1B).

2.12.5 *In vitro* dephosphorylation assays

GST-capsid was transiently expressed in COS cells and labeled with ^{33}P-orthophosphate as described above. Cells were lysed on ice with lysis buffer (1% NP-40, 350 mM NaCl, 2 mM EDTA, 50 mM Tris Cl (pH 7.5)) plus phosphatase inhibitors. Radiolabeled GST-capsid was isolated on glutathione-Sepharose beads at 4 ^{0}C. The beads were washed three times with cold lysis buffer and resuspended in solution containing 50 mM Tris Cl, 2mM CaCl2 (pH 7.5). Aliquots of the beads were incubated with one microgram of purified phosphatases (protein phosphatase 1A (PP1A), PP1B, PP2A, PP2B, or calf intestinal alkaline phosphatase) for 16 hours at 37 °C. Samples were separated by SDS-PAGE and transferred to PVDF membranes before analysis on a

Phosphorimager. Total levels of GST-capsid were determined by probing membranes with rabbit anti-GST antibodies followed by ECL detection.

Chapter 3. Capsid dependent role of the E2 signal peptide in virion assembly

Data from this chapter were published in "Law *et al.* 2001. Rubella virus E2 signal peptide is required for perinuclear localization of capsid protein and virus assembly. J. Virology. 75(4):1978-83"

3.1 Overview

RV is a member of the family Togaviridae that also includes the well-characterized alphaviruses. Togaviruses exhibit conserved structural virion features, genome organization and gene expression pathways (for review (Frey, 1994; Murphy, 1980)). However there are a number of significant differences between the assembly pathways of alphaviruses and RV. For example, preformed nucleocapsids are readily observed within the cytoplasm of alphavirus infected cells. Interactions between nucleocapsids and the cytoplasmic domains of viral glycoproteins are believed to drive alphavirus budding at the plasma membrane. In contrast, the assembly of RV nucleocapsids is membrane-dependent and coincides with virus budding at the Golgi complex. It has been proposed that the difference between alphaviruses and RV in the processing of capsid protein from the polyprotein precursor contributes to the distinct virus assembly pathways (for review (Frey, 1994)).

A second important difference between alphaviruses and RV is that the RV capsid does not have autoprotease activity. Instead, RV capsid is processed by the host signal peptidase, cleaving the polyprotein at the carboxyl terminus of the E2 SP. As a result, the 23 amino acid residue E2 SP is retained as part of the RV capsid carboxyl terminus (Hobman and Gillam, 1989; Suomalainen et al., 1990) (Figure 1.4). The E2 SP is predicted to form a hydrophobic α-helical domain that mediates membrane association of capsid. The precise nature of this interaction is not understood, but a previous study reported that approximately 50% of nascent capsid became stably associated with membranes (Suomalainen et al., 1990). In addition to mediating membrane association with capsid, the E2 SP affects capsid intracellular localization suggesting targeting as its essential role in capsid function.

Aside from functioning as a membrane anchor for capsid (Suomalainen et al., 1990), the E2 SP appears to have additional roles in virus assembly. Indeed, previous work has shown that the E2 SP is essential for correct targeting of the protein. Deletion of this domain from capsid, results in the accumulation of capsid in cytoplasmic puncta that do not resemble ER or Golgi elements (Baron et al., 1992). Based on the topology of structural proteins, it is predicted that the membrane-spanning domains of structural proteins (i.e. E2 SP, E2 TM, E1 SP and E1 TM) are in close proximity within the virus envelope (Figure 1.4). Work from this lab is consistent with the notion that interactions between the E2 TM and E1 TM are essential for mediating binding between glycoproteins E2 and E1 (Garbutt et al., 1999). By analogy, it is possible that the E2 SP is involved in lateral interactions between capsid and glycoproteins E2 and/or E1. Accordingly, replacement of the glycoprotein E2 TM by the TM from the VSV G protein, not only affected the targeting of E2, but also the targeting of capsid in that the latter protein was exhibit largely cytoplasmic staining (Garbutt et al., 1999). This suggests that the virus glycoproteins, in particular E2, are essential for targeting RV capsids to the virus assembly site. I hypothesized that the E2 SP acts as more than a membrane anchor to actively target capsid to the virus budding site. Furthermore, the predicted lateral interaction between membrane-spanning domains may be essential for driving virion formation via coordinating capsid-glycoproteins interaction.

3.2 E2 signal peptide is essential for virus particle secretion

In order to test this hypothesis, the E2 SP at the carboxyl terminus of capsid was replaced with SPs from host and viral glycoproteins. The SPs from the membrane proteins CD8 and VSV G were chosen for these studies. The sequences of these domains are shown in Figure 3.1. Mammalian cells that express RV structural proteins have been shown to assemble and secrete rubella virus-like particles (RLPs) that are very similar to infectious RV virions in terms of morphology and antigenicity (Hobman et al., 1994b). Accordingly, RLPs have proved to be a useful model system with which to study the parameters of RV assembly (Hobman et al., 1994b). In order to assay the importance of the E2 SP in virus assembly and secretion, COS cells were transiently co-transfected with plasmids encoding the RV glycoproteins, E2 and E1, and capsid proteins with (CapE2SP) or without (CapΔSP) E2 SP respectively (Figure 3.1). RLP secretion was determined using an immunoblot-based assay (Garbutt et al., 1999) (Figure 3.2A).

Forty-eight hours post-transfection, culture media were precleared by centrifugation at 10,000 x *g* to remove cell-associated material followed by a second centrifugation of 100,000 x *g* for 1 hour to pellet RLPs. When cells were co-transfected with plasmids encoding CapE2SP and E2E1, capsid was detected in the cell lysates and 100,000 *g* media pellets (Figure 3.2B, lanes 1 and 2). The presence of capsid in the 100,000 *g* media pellets indicated that RLPs were assembled and secreted from the cells (Garbutt et al., 1999). Cells expressing E2E1 and capsid lacking the E2 signal peptide (CapΔSP) produced high levels of capsid protein that were detectable in cell lysates, but not in the 100,000 *g* media pellets (Figure 3.2B, lanes 7 and 8). These results indicate that

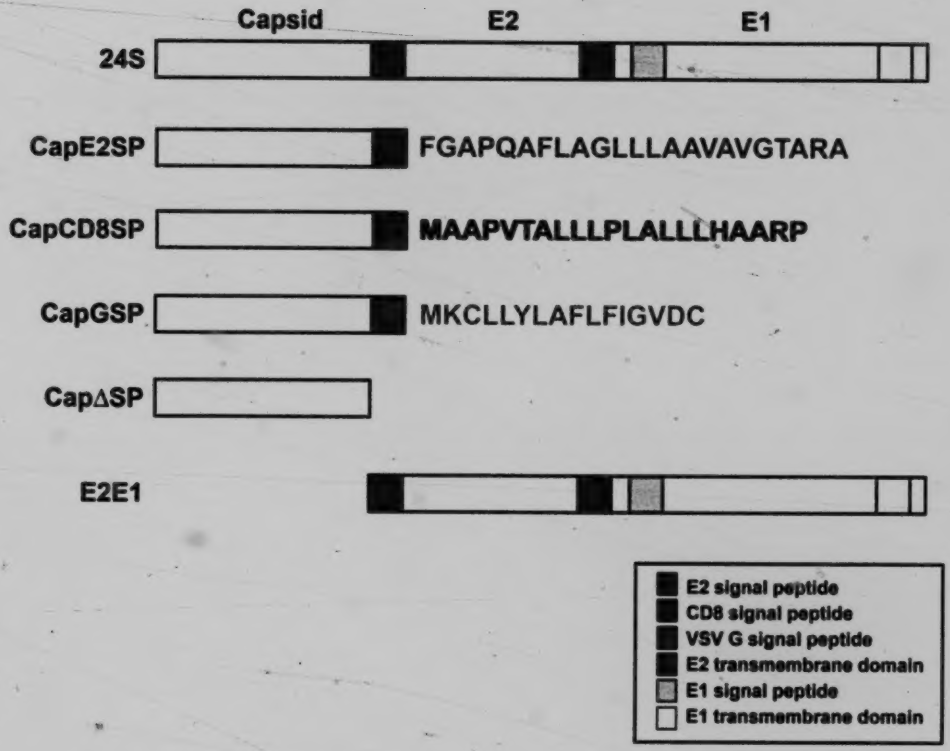

Capsid
E2
E1
24S
CapE2SP
FGAPQAFLAGLLLAAVAVGTARA
CapCD8SP
MAAPVTALLLPLALLLHAARP
CapGSP
MKCLLYLAFLFIGVDC
CapΔSP
E2E1
E2 signal peptide
CD8 signal peptide
VSV G signal peptide
E2 transmembrane domain
E1 signal peptide
E1 transmembrane domain

Figure 3.1. Schematic of RV protein expression constructs. The 24S cDNA encodes all three RV structural proteins in the order capsid-E2-E1. The rest of the constructs are named according to the heterologous domains encoded. The CapE2SP encodes amino acid residues 1-300 of wild type capsid protein including the E2 signal peptide attached to its carboxyl terminus. The CapCD8SP and CapGSP constructs encode amino acid residues 1-277 of capsid protein fused to the CD8 or VSV G signal peptides respectively. The amino acid sequences of the SPs from E2, CD8 and VSV G are shown next to each construct. The CapΔSP encodes the amino acid residue 1-277 of wild type capsid without the E2 signal peptide at its carboxyl terminus. The E2E1 construct encodes RV glycoproteins E2 and E1. Signal peptides and transmembrane domains are indicated by colored boxes.

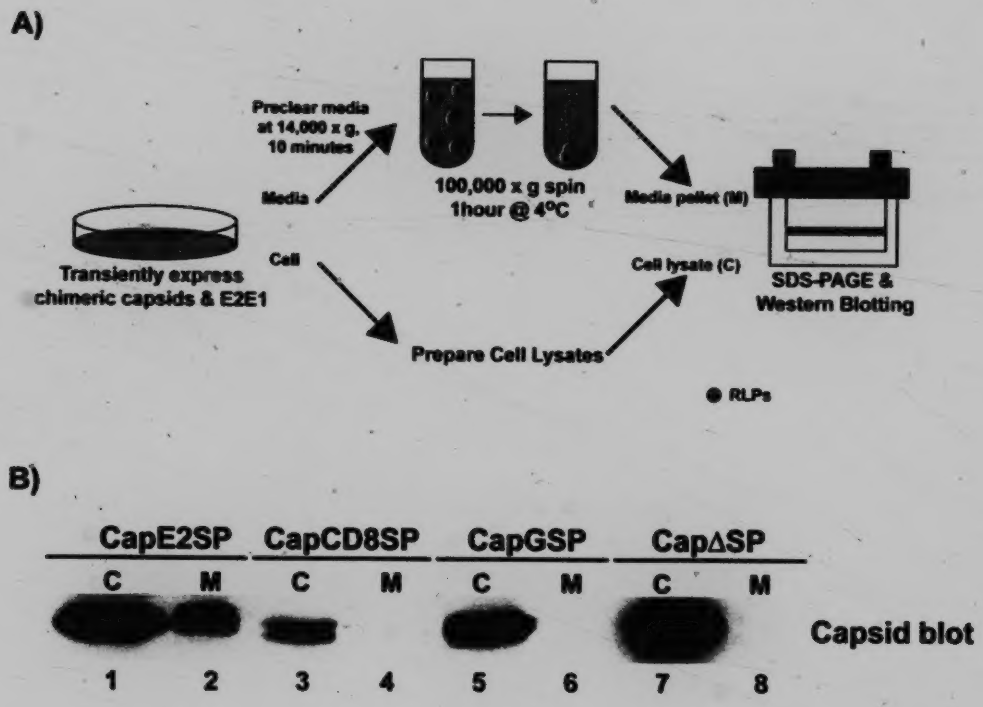

A)
Preclear media at 14,000 x g, 10 minutes
Media
Cell
Transiently express chimeric capsids & E2E1
100,000 x g spin 1hour @ 4°C
Media pellet (M)
Cell lysate (C)
SDS-PAGE & Western Blotting
Prepare Cell Lysates
● RLPs
B)
CapE2SP
CapCD8SP
CapGSP
CapΔSP
C
M
C
M
C
M
C
M
Capsid blot
1
2
3
4
5
6
7
8

Figure 3.2. The E2 signal peptide is required for RLP secretion. Capsid constructs contains E2, CD8 or VSVG signal peptides were co-transfected with the E2E1 expression plasmid into COS cells. A) Schematic of the RLP secretion assay. Forty-eight hours post-transfection, media from the transfected cells were pre-cleared of cell-associated material and the resulting supernatants were subjected to centrifugation at 100,000 x *g* to pellet RLPs (M). Cell lysates (C) were also prepared from the transfected cells to confirm the expression of each capsid chimera. Lysates (C) and membrane pellets (M) were subjected to SDS-PAGE, transferred to PVDF membranes and immunoblotted with anti-capsid antibody followed by ECL detection. B) A representative result of the RLP secretion assay. COS cells transiently co-expressing various capsid constructs with different signal peptides and E2E1 were subjected to RLP secretion assay. Capsid proteins that are incorporated into secreted RLPs were detected in the membrane pellet fractions (M).

the presence of a signal peptide on the capsid protein is required for RLP assembly and/or secretion.

3.3 Heterologous signal peptides do not support RLP secretion

The experiments shown in Figure 3.2B demonstrated that deletion of the E2 SP from capsid abrogates secretion of RLPs, however, they did not address whether this domain functions simply as a membrane anchor or if it has additional roles in virus assembly and/or secretion. If the former were true, signal peptides from other transmembrane glycoproteins should be able to functionally replace the E2 SP. To determine if capsids containing heterologous signal peptides could function in virus assembly, the signal peptides from two other type I membrane glycoproteins, CD8 and VSV G, were fused onto the carboxyl terminus of RV capsid, in place of the E2 SP to create CapCD8SP and CapGSP respectively (Figure 3.1).

The CD8 and VSV G signal peptides are 21 and 16 amino acids in length respectively whereas the E2 SP is 23 amino acids long (Figure 3.1). However, all three of these hydrophobic peptides are theoretically long enough to span the ER and Golgi membranes to function as transmembrane domains (Bretscher and Munro, 1993). COS cells were transiently co-tranfected with plasmids encoding these capsid constructs and a plasmid encoding RV E2 and E1 and RLP secretion was assayed as described above. RLP secretion was observed only in cells expressing CapE2SP and E2E1 (Figure 3.2B, lane 2). Importantly, capsid proteins were detected in all cell lysates indicating that these chimeric capsids are stably expressed (Figure 3.2B). These data suggest that the signal peptides of CD8 and VSV G cannot functionally replace the E2 signal peptide to support

RLP secretion. However, the experiments do not address whether the E2 SP is required for virus particle assembly and/or secretion.

3.4 CD8 and VSV G signal peptides are sufficient to mediate capsid membrane association

There are two obvious possibilities to account for the failure of cells expressing CapCD8SP and CapGSP to secrete RLPs. The first possibility is that these capsid chimeras do not stably associate with membranes and are therefore unable to function in RLP assembly. An alternative explanation is that the E2 SP interacts with other viral components in a sequence-specific manner during assembly and/or secretion. To address the first hypothesis, I employed a previously described membrane co-pelleting assay to determine whether or not the chimeric capsid proteins could stably associate with membranes (Suomalainen et al., 1990). Briefly, the capsid proteins were synthesized using the *in vitro* coupled transcription/translation system in either the presence or absence of microsomes. After the synthesis reactions, the reaction mixtures were extracted with sodium carbonate to separate integral membrane proteins from soluble and peripheral membrane proteins. Samples were then subjected to centrifugation through a sucrose cushion. Under these conditions, only capsids that are stably associated with microsomes, were expected to be recovered in the pellet fractions.

Figure 3.3A illustrates the results from a representative membrane-copelleting assay. Quantitation of the capsid bands by densitometry indicated that synthesis in the presence of microsomes resulted in a 3 to 4 fold increase in the proportion of membrane-associated capsid proteins, regardless of which SP was attached to the capsid. In contrast, the fraction of CapΔSP in the pellet, was not significantly affected by the presence of

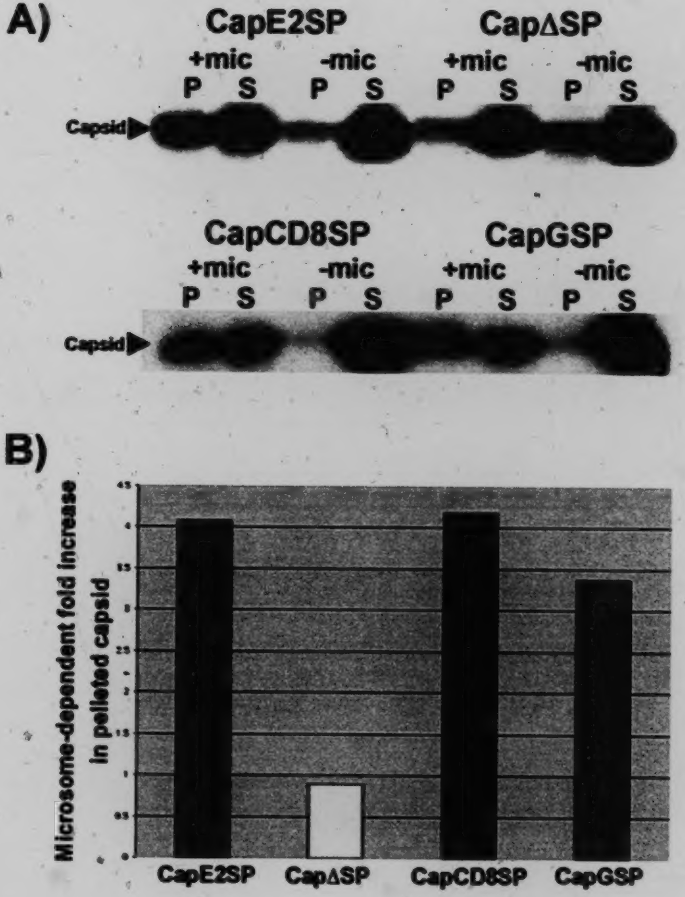


Figure 3.3. Capsids with heterologous signal peptides stably associate with membranes *in vitro*. Capsid constructs with E2, CD8 or VSV G signal peptides were synthesized *in vitro* in the presence or absence of canine pancreatic microsomes (+/- mic). Samples were extracted with 50 mM sodium carbonate (pH 11.5) and then membranes were pelleted through a sucrose cushion using a Beckman airfuge set at 25 lb/in^2. The supernatant (S) and the pelleted membranes (P) were subjected to SDS-PAGE and fluorography. A) Fluorographs from a representative experiment are shown. B) Graphical representation of membrane-associated capsids (average of two independent experiments). Y-axis represents the fold increase in the proportion of pelleted capsids when translated in the presence of microsomes relative to the proportion of pellet capsids when translated in the absence of microsomes.

microsomes (Fig. 3.3B). These results are similar to those of Suomalainen *et al.* (Suomalainen et al., 1990) who showed that removal of the E2 SP from capsid proteins resulted in a 3.5 fold decrease of capsid association with microsomes under the same conditions. Based on the observation that heterologous SPs support capsid membrane association but not RLP secretion, I conclude that the E2 SP performs additional functions during RLP assembly and/or secretion.

3.5 The role of E2 signal peptide in virus particle assembly

In light of work from our lab showing that alteration of the E1 membrane spanning or cytoplasmic domains blocks secretion but not assembly of RLPs (Garbutt et al., 1999), it is important to determine whether the E2 SP is required at a pre- or post-virus assembly step. Transient expression of CapE2SP in CHO cells stably expressing E2 and E1 (CHO-E2E1 (Hobman et al., 1992)) resulted in the formation of RLPs that can be visualized by electron microscopy (Fig. 3.4A, arrowheads). These structures were readily visible in the Golgi complex of cells expressing RV structural proteins by this method whether they are secreted or not (Garbutt et al., 1999). The same assay was used to determine if co-expression of mutant capsid proteins in CHO-E2E1 cells resulted in assembly but not secretion of RLPs.

CHO-E2E1 cells were transfected with the four capsid constructs (Figure 3.1) and 48 hours post-transfection, cells were processed for routine morphology as described (Garbutt et al., 1999). Transfection efficiencies were monitored by indirect immunofluorescence using rabbit anti-capsid antibodies. About 10% transfection efficiency was routinely achieved for each capsid construct. At least 80 sections from each sample were examined for the presence of RLPs in the Golgi cisternae or associated

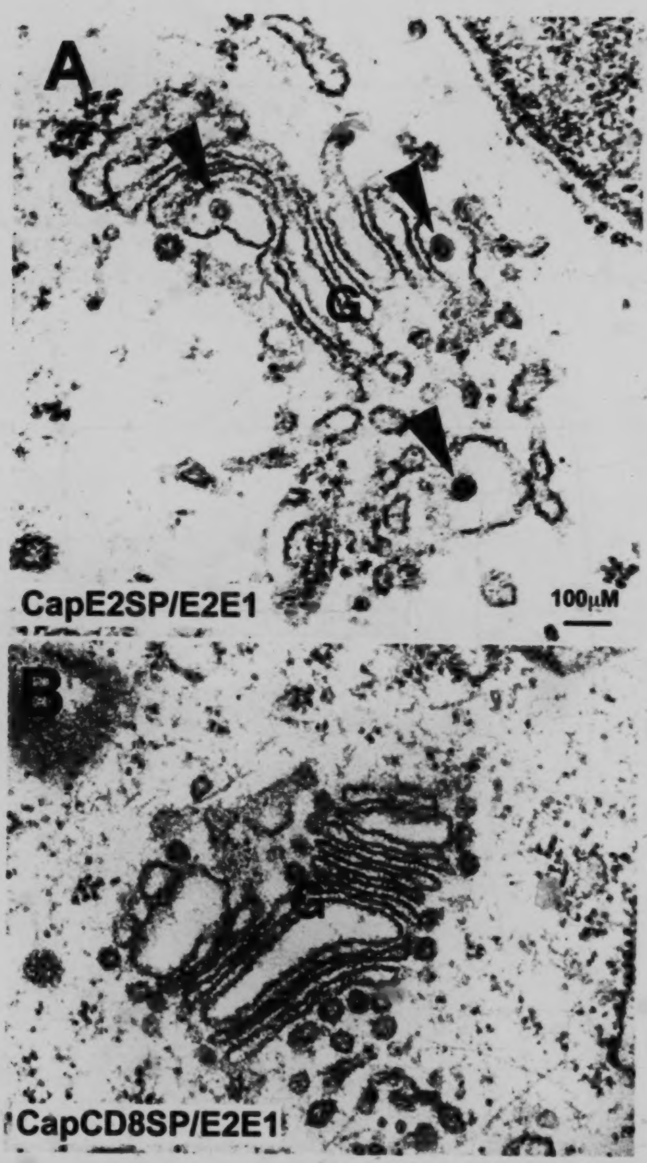


Figure 3.4. The E2 signal peptide is required for assembly of virus particles. CHO-E2E1 cells were transfected with expression vectors encoding different capsid proteins and 48 hours post-transfection, cells were prepared for routine morphology by embedding in Epon. A) Electron micrograph of CHO-E2E1 cells transfected with CapE2SP. RLPs (arrowheads) can be seen in the Golgi complex (G) of these cells. No RLPs were observed in the Golgi complex of CapCD8SP transfected cells (B). Bar in panel A = 100 μm.

vesicles. In CHO-E2E1 cells transfected with CapE2SP (Figure 3.4A), RLPs were seen in the Golgi complex of approximately 10% of the cells examined and therefore the proportion of cells containing RLPs correlated with the transfection rate. No RLPs were detected in CHO-E2E1 cells transfected with CapCD8SP (Fig. 3.4B). Among the more than 100 sections analyzed, a single RLP was detected in a CHO-E2E1 cell transfected with CapGSP (Table 3.1). Together, these data argue that the E2 SP does not simply function as a membrane anchor, but that it is also required for efficient virus assembly.

3.6 The E2 signal peptide is required for targeting capsid to the site of virus budding

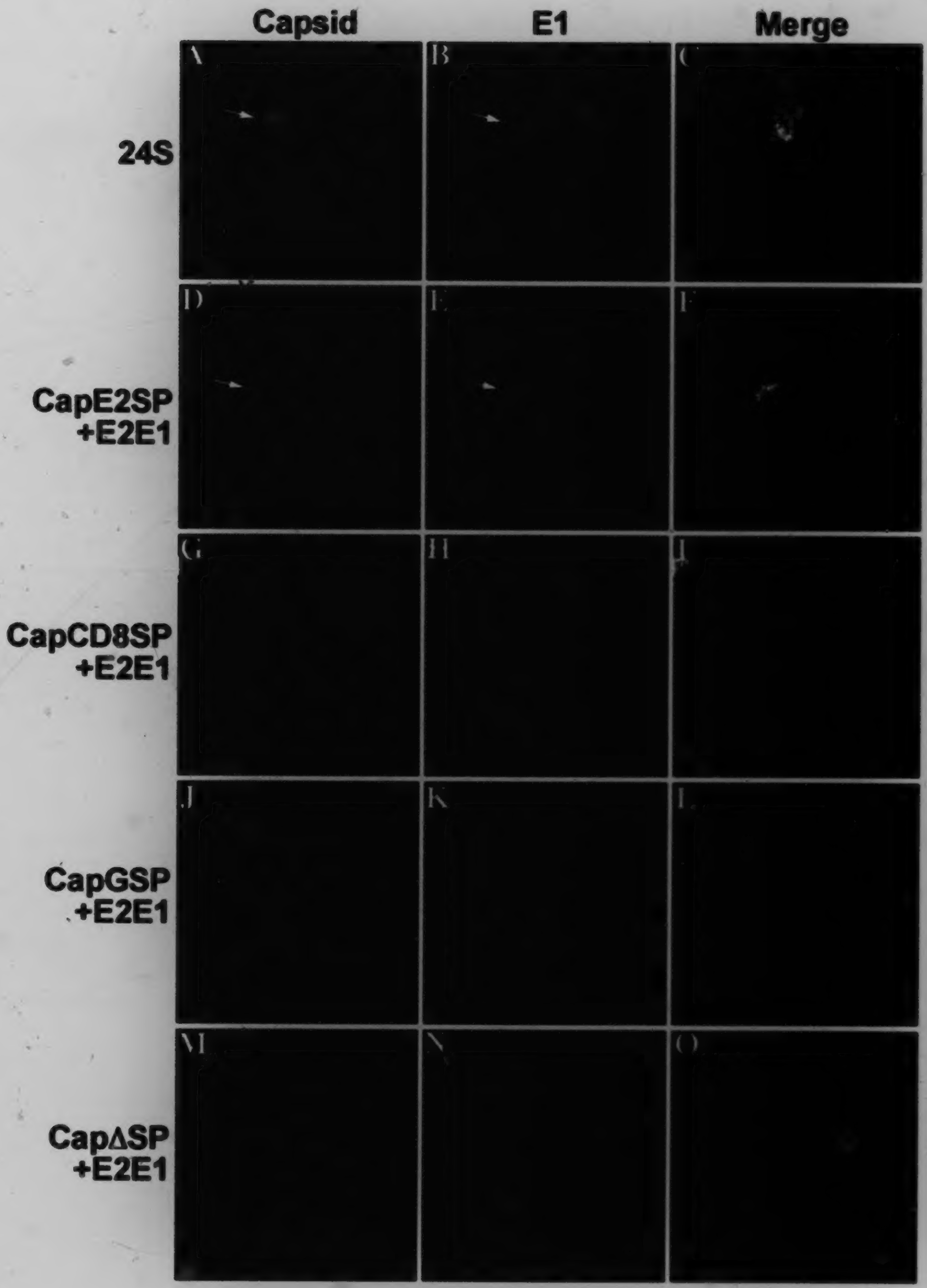
Transport of RV structural proteins to the perinuclear region is required for efficient assembly of RLPs (Garbutt et al., 1999). Accordingly, failure to properly target structural proteins to the virus assembly site would be expected to result in impaired RLP assembly. Therefore, I decided to examine the subcellular localization of the different capsid constructs to determine if the E2 SP was required for transport of capsid to the same perinuclear region where glycoproteins E2 and E1 accumulate. COS cells grown on coverslips were transiently transfected with plasmids encoding capsid, E2 and E1 (24S) or E2E1 plus different capsid constructs (Figure 3.1). Samples were processed for double-label indirect immunofluorescence 24 hours post-transfection using a Zeiss 510 confocal microscope.

Whether cells were transfected with a single plasmid encoding capsid, E2 and E1 (24S), or two plasmids encoding capsid and the viral glycoproteins separately (CapE2SP+E2E1), limited areas of colocalization between capsid and E1 were observed in the juxtanuclear region (Figure 3.5 A-F arrows). These data were not surprising and

	Number of Golgi-containing fields screened	Number of Golgi containing RLPs
CapE2SP	80	7
CapCD8SP	133	0
CapGSP	105	1

Table 3.1. Number of Golgi containing RLPs

Figure 3.5. Capsids with heterologous signal peptide do not co-localize with E1 in the juxtanuclear region. COS cells were transfected with expression vectors that encode 24S (Capsid+E2+E1), CapE2SP, CapGSP, CapCD8SP, or CapΔSP and E2E1. Cells were fixed and permeabilized using methanol, then double-labeled with rabbit anti-capsid antibodies (7W7) (A, D, G, J and M) and mouse anti-E1 antibodies (B2) (B, E, H, K and N). Samples were then examined by confocal microscopy. Optical sections (0.5 μm) from the X-Y planes are shown. Primary antibodies were detected with FITC-conjugated donkey anti-rabbit IgG and Texas Red-conjugated goat anti-mouse IgG. The FITC channel is shown on the left (A, D, G, J, M), and the Texas Red channel is shown in the middle (B, E, H, K, N). The merged images are shown on the right (C, F, I, L, O). Arrows indicate regions where limited colocalization between capsid and E1 occurs.


Capsid
E1
Merge
A
B
C
24S
D
E
F
CapE2SP +E2E1
G
H
I
CapCD8SP +E2E1
J
K
L
CapGSP +E2E1
M
N
O
CapΔSP +E2E1

suggest that a pool of E1 and capsid were accumulating at the same intracellular localization (Hobman et al., 1990; Hobman et al., 1994a). Moreover, the lack of complete colocalization between capsid and E1 was expected since a significant proportion of capsid but not E1 or E2 associates with the mitochondria (Beatch and Hobman, 2000; Lee et al., 1999). In contrast, CapCD8SP, CapGSP and CapΔSP exhibited punctate or reticular staining throughout the cytoplasm and did not colocalize with E1 (Figure 3.5 G-O). It has been shown previously that the distributions of CapCD8SP and CapGSP partially overlapped with the ER protein calnexin in transfected COS cells, indicating pools of these proteins are localized to membranes of the ER (Duncan et al., 2000). Collectively, these data indicate that the E2 SP is required for recruitment of capsid to the perinuclear region, and furthermore, this process requires the co-expression of the virus glycoproteins E2 and E1.

3.7 Summary

The retention of the E2 SP on the carboxyl terminus of RV capsid protein is unique among Togaviruses. In this section, I have shown that this hydrophobic domain is essential for mediating capsid membrane association. Replacement of E2 SP with the SPs of CD8 or VSV G, did not affect membrane association of capsid, however, RLP assembly, and consequently secretion of virus particles, was abrogated. Subsequent analysis revealed that the chimeric capsid proteins exhibited different subcellular localization patterns. Specifically, the chimeric capsid proteins were not targeted to the perinuclear region of the cell where virus assembly occurs. These results suggest that the E2 SP contains information needed to properly target capsid to the virus assembly site. At this point, the mechanisms of how E2 SP functions in targeting of capsid to the budding site are not known. However, based on the current data and previous results showing that capsid transport to the virus budding site is glycoprotein-dependent (Baron et al., 1992), it seems reasonable to assume that the E2 SP is involved in capsid-glycoprotein interactions, possibly via the lateral interaction with other membrane spanning domains of the glycoproteins.

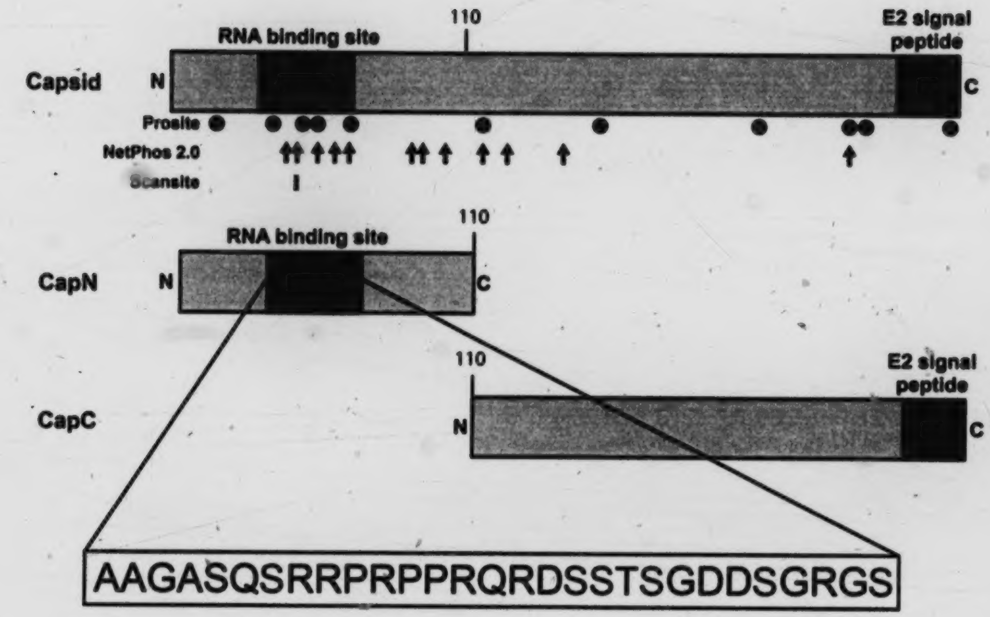
Chapter 4 . Role of capsid phosphorylation in virus replication

Part of the data from this chapter were published in "Law *et al.*, 2003. Phosphorylation of rubella virus capsid regulates its RNA binding activity and virus replication." J. Virology. 77(3): 2010-20." Figure 4.7 was provided by Jason Everitt (University of Alberta). Figures 4.8 and 4.9 were provided by Dr. Wen-Pin Tzeng (Georgia State University).

4.1 Overview

While it has long been known that the RV capsid is phosphorylated prior to virus assembly (Garbutt et al., 1999; Marr et al., 1991), the significance of this post-translational modification has been overlooked in Togaviruses. However, in a limited number of other viruses including hepatitis B virus, hepatitis C virus and retroviruses, phosphorylation of capsid proteins is known to be important for viral replication (Cartier et al., 1999; Gazina et al., 2000; Kock et al., 2003; Lan et al., 1999; Lu and Ou, 2002). Although the replication and life cycles of these viruses are distinct from RV, all of their capsid proteins share the function of binding the viral genome during formation of the nucleocapsid core. Therefore, I set out to determine the role of capsid phosphorylation in RV replication.

Analysis of the capsid sequence revealed that a high number of potentially phosphorylated serine and threonine residues were clustered within the RNA binding site (Figure 4.1). This region also overlaps with the binding sites for two host encoded proteins, p32 and Par-4 (Beatch, 2004). The significance of Par-4 binding to capsid remains to be determined and will not be discussed here. However, the capsid-p32 interaction is particularly intriguing because binding of this host protein is known to regulate the activities of multiple kinases as well as the phosphorylation state and RNA binding activities of splicing factors such as ASF/SF2 (Petersen-Mahrt et al., 1999; Robles-Flores et al., 2002). Together, these observations led me to hypothesize that phosphorylation is important for regulating capsid-RNA interaction.



110
RNA binding site
E2 signal peptide
Capsid
N
C
Prosite
NetPhos 2.0
Scansite
110
RNA binding site
CapN
N
C
110
E2 signal peptide
CapC
N
C
AAGASQSRRPRPPRQRDSSTSGDDSGRGS

Figure 4.1. Schematic of RV capsid protein constructs. The full length RV capsid is comprised of 300 amino acid residues. CapN encodes the amino terminal 110 amino acid residues of the capsid protein. The genomic RNA binding site is highlighted by a blue box. CapC encodes an initiator methionine followed by amino acids 107 to 300 of capsid including the E2 signal peptide (red box). The relative positions of potential phosphorylation sites are denoted with dots, arrows and bars based on their respective algorithms (see text): Prosite (dot), Netphos 2.0 (arrow) and Scansite (bar). There are a total of 18 potentially phosphorylated serine and threonine residues throughout capsid. Seven of these residues are clustered within the RNA binding site. The sequence of the RNA binding region is shown in the expanded white box and potentially phosphorylated residues are highlighted in red.

4.2 Identification of phosphorylated amino acid residues in capsid

In order to determine if capsid phosphorylation is important for virus replication, it was necessary to first map the phosphorylated amino acid residue(s) within this protein. Analysis of the capsid sequence using Prosite, Netphos and Scansite algorithms revealed the presence of 19 potentially phosphorylated serine and threonine residues. Interestingly, seven of them are clustered within the RNA binding site (Figure 4.1).

Capsid constructs encoding the amino-terminal (CapN) and carboxyl-terminal regions (CapC) of capsid were used to crudely map the region of capsid that contains phosphorylated residues (Figure 4.1). The constructs were transiently expressed in COS cells, which were then labeled for 12 hours with [^{33}P]-orthophosphate. Capsid proteins were recovered by immunoprecipitation and analyzed by SDS-PAGE and fluorography. Both full length capsid and CapN (which encodes the first 110 amino acid residues of capsid including the RNA-binding site) efficiently incorporated radiolabeled phosphate (Figure 4.2A, upper panel lanes 1 and 2). In contrast, CapC (which encodes amino acid residues 107 to 300) was not labeled with radioactive phosphate under these conditions (Figure 4.2A upper panel, lane 3). Immunoblot analysis confirmed that the CapC construct was stable and adequately expressed in the transfected cells (Figure 4.2A, lower panel). Accordingly, the most logical interpretation of these results is that all of the phosphorylated residues in capsid and/or amino acid residues that are necessary for phosphorylation, are located within the first 110 amino acid residues of the protein. Conversely, the kinase that normally phosphorylates amino acid residues in the carboxyl terminal two thirds of the protein may not recognize the truncated capsid (CapC) as a substrate. However, results shown below argue against this possibility.

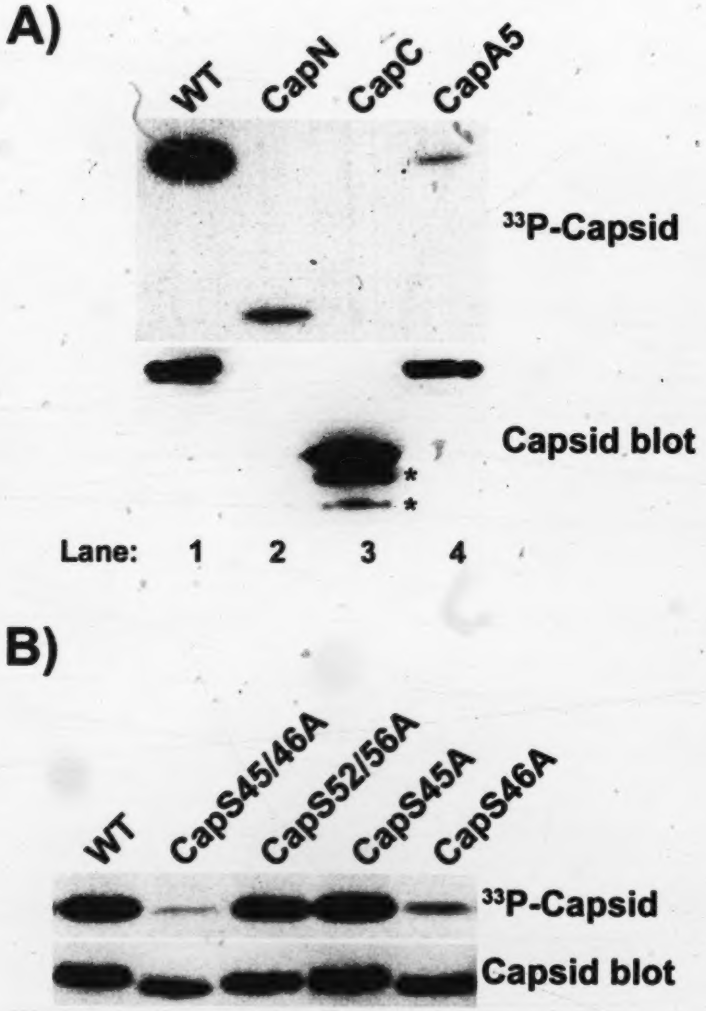

A)
WT
CapN
CapC
CapA5
33P-Capsid
Capsid blot
*
*
Lane: 1 2 3 4
B)
WT
CapS45/46A
CapS52/56A
CapS45A
CapS46A
33P-Capsid
Capsid blot

Figure 4.2. Serine 46 in the RNA binding site is essential for capsid phosphorylation. A) Plasmid encoding RV capsid (WT) and capsid deletion constructs were transfected into COS cells and 24 hours post-transfection, cells were incubated with media containing [^{33}P]-orthophosphate for 12 hours. Cells were lysed and subjected to radioimmunoprecipitation using rabbit anti-capsid antibodies, SDS-PAGE and fluorography (upper panel). Total capsid protein expression levels were monitored by immunoblotting of cell lysates on separate gels (lower panel). Two unidentified bands (*) are present in the CapC lane. The failure to detect protein expression of CapN in this experiment by immunoblotting is due to the fact that the fragment ran off the gel. B) The phosphorylation levels of capsid and different alanine mutants were compared.

Within the first 110 amino acid residues of capsid, most of the potentially phosphorylated residues are located within the RNA binding site (Figure 4.1). In order to identify which of these residues are critical for capsid phosphorylation, site-directed mutagenesis was used. All constructs generated by site-directed mutagenesis together with their phenotypes are summarized in Table 4.1. These constructs were expressed in transfected COS cells that were subsequently radiolabeled with phosphate as described above. Of all the constructs tested, CapA5, in which serines 45, 46, 48, 52 and 56 are replaced with alanine residues, had the most dramatic reduction in the level of phosphorylation (Figure 4.2A). Quantitation of the radioimmunoprecipitates with a phosphorimager revealed that CapA5 incorporated less than 2% of the labeled phosphate incorporated by wild type capsid. This suggested that the majority of phosphorylation occurs within the RNA binding site of capsid. Next, a series of single and double alanine mutants were constructed and analyzed in the same manner to determine which of the serine/threonine residues in the RNA-binding site become phosphorylated. Mutants that included the changing of serine 46 to alanine (e.g. CapS45/46A and CapS46A) have the most dramatic reduction of capsid phosphorylation (Figure 4.2B). The level of phosphorylation in S46A was comparable to CapA5. In contrast, mutation of serines 45, 52, and 56 (Figure 4.2B) to alanines had little or no effect on capsid phosphorylation. Low levels of ^{33}P-labeling were observed in all cases, which could mean that other amino acid residues in capsid are phosphorylated albeit at much lower levels (see later Section 4.8). These results indicate that serine 46 of capsid is critical for normal capsid phosphorylation.

Alanine mutations

Construct	Mutation(s)$^{+}$	Phosphorylation level	RNA binding activity
CapT8A	T8	++	ND
CapS34A	S34	++	ND
CapA3	T47, S48, S52	-^	+
CapA5	S45, S46, S48, S52, S56	-	+
CapA4-1	S45, S46, S48, S52	-	+
CapA4-2	T47, S48, S52, S56	++	+
CapP5	S45, S46, T47, S48, S52	-	+
CapP4	S45, S46, T47, S48	-	+
CapP3	S45, S46, T47	-	+
CapS45/46A	S45, S46	-	+
CapS52/56A	S52, S56	++	-
CapS45A	S45	++	-
CapS46A	S46	-	+
CapT47A	T47	++	+

Asparate/ Asparagine/ Glutamate mutations

Construct	Mutation(s)$^{+}$	Phosphorylation level	RNA binding activity
CapP6D	S45, S46, T47, S48, S52, S56	-	+
CapP5D	S45, S46, T47, S48, S52	-	+
CapP3D	S45, S46, T47	+	+
CapP3E	S46, T47, S48	-*	+
CapS46N	S46	-	+
CapS46D	S46	++	-
CapS46E	S46	++	+
CapT47E	T47	++	-

RNA binding site

$^{+}$MASTTPITMEDLQKALEAQSRALRAG$\boxed{\text{LAAGASQSRRPRPPRQRDSSTSGDDSGRDS}}$

8 34 45464748 52 56

^ Higher than CapS46A/CapA5

* Slight increase compared to CapS46A

Table 4.1. List of recombinant capsid constructs. All of the recombinant capsid constructs generated in this study and their relative phosphorylation levels and RNA binding activities are summarized in this table. The amino acid sequence of the amino-terminus of RV capsid is shown in the footnote of the table. The RNA binding site of capsid is boxed and each of the potentially phosphorylated residues is denoted with the amino acid residue number underneath. Three different levels of phosphorylation were observed: (++) represents the level of phosphorylation observed in the wild type capsid, (-) represents the lowest level of phosphorylation observed among capsid mutants (e.g. CapS46A) and (+) represents an intermediate level of phosphorylation. Similarly, the RNA binding activities of capsid proteins were classified as high (+) or low (-) as determined using the *in vitro* RNA binding assay. ND; not determined.

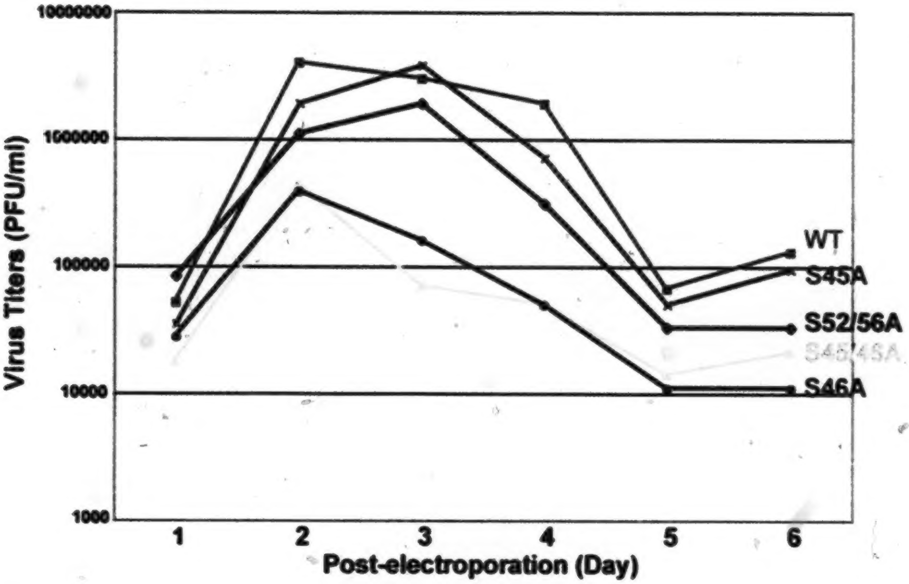
4.3 Capsid phosphorylation is important for virus replication

To examine the importance of capsid phosphorylation in virus replication, the serine-alanine mutants were expressed in the context of an infectious RV clone (Yao and Gillam, 1999). Capped RV genomic RNAs from the wild type M33 (WT) and capsid mutant strains were synthesized *in vitro* and equal amounts of RNA were electroporated into BHK cells. The media from transfected cells were collected at daily intervals and virus titers were determined by plaque assay using RK-13 cells. The titers for all viruses peaked between two and three days post-electroporation (Figure 4.3A). However, the peak virus titers of hypophosphorylated capsid-containing viruses (S45/46A and S46A) were 10 fold lower than virus strains that encode normally phosphorylated capsids (WT and S45A). The difference in virus titers was more dramatic at day 3 post-electroporation. The titer of S46A virus was as much as 60 fold lower than the wild type virus (Figure 4.3A). This suggests that capsid phosphorylation affects the amount of infectious virus secreted, possibly by affecting virus replication (see Section 4.6).

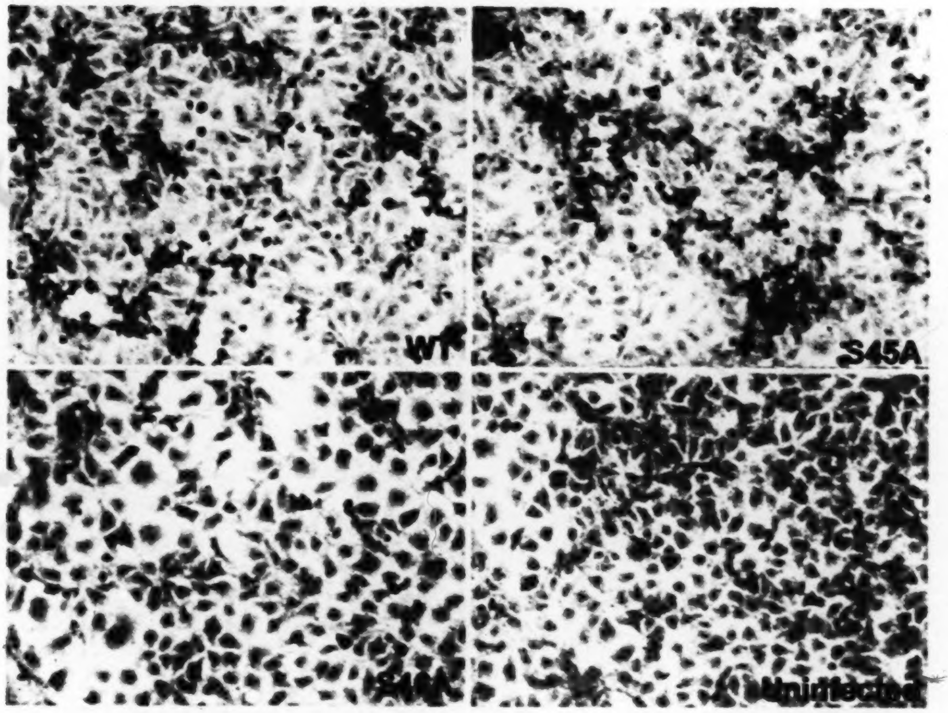
In addition to the defect in virus replication, the cytopathic effects of these mutant viruses were markedly reduced. RK-13, a cell line that is exquisitely sensitive to RV cytopathic effects, was infected with M33 and capsid mutant strains at the same multiplicity of infection (MOI) and then examined for cytopathic effects after two to four days. Figure 4.3B clearly shows that the S46A mutant strain is less cytopathic than M33 or S45A mutant strains. These results demonstrate that although capsid phosphorylation is not essential for virus replication, abrogation of this process significantly impairs the secretion of infectious virus and reduces cytopathic effects.

Figure 4.3. Capsid phosphorylation is important for virus replication. A) Equal amounts of viral transcripts encoding either the wild type M33 or mutant genomes were electroporated into BHK cells. Each day post-electroporation, media were collected to quantitate the amount of secreted virus by plaque assay using RK-13 cells. B) RV strains that harbor hypophosphorylated capsids are less cytopathic. RK-13 cells were infected with M33 (WT), S45A or S46A virus strains at a MOI of 0.25. Four days post-infection cells were stained with crystal violet and photographed. Cells exhibiting cytopathic effect are rounded and darkly stained.

A)


10000000
1000000
100000
10000
1000
Virus Titers (PFU/ml)
1
2
3
4
5
6
Post-electroporation (Day)
WT
S45A
S52/56A
S45/46A
S46A

B)


WT
S45A
S46A
Uninfected

4.4 Capsid phosphorylation negatively regulates its RNA binding activity

One of the functions of capsid during virus assembly is to package genomic RNA into nucleocapsids. Since the majority of phosphorylation occurs in the RNA-binding site of capsid, I reasoned that this post-translational modification may regulate binding of genomic RNA and subsequent nucleocapsid assembly. Therefore, the abilities of capsid phosphorylation mutants to bind viral RNA were tested using an *in vitro* RNA binding assay (Liu et al., 1996b). Capsid proteins transiently expressed in COS cells were immunoprecipitated, separated by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated with a radiolabeled RV-specific RNA probe that includes the capsid-binding packaging signal (Liu et al., 1996b). These experiments revealed that hypophosphorylated capsids (S45/46A and S46A) bound RNA more efficiently than wild type capsid or mutants (S52/56A and S45A) that retained normal levels of phosphorylation (Figure 4.4A).

Initially, the inability of wild type capsid to interact with RNA was somewhat puzzling since at some point the RV capsid must interact with viral RNA to form nucleocapsids. In addition, a similar assay employed by Liu *et al.* was used to map the sites of interaction between capsid and genomic RNA (Liu et al., 1996b). A critical difference between their experiments and mine was that in the former study, the capsid protein used for the binding studies was isolated from virions whereas my assay employed capsids isolated from transfected cells. Similar to Liu *et al.*, I was able to demonstrate that virion-derived wild type capsids efficiently bound viral RNA (Figure

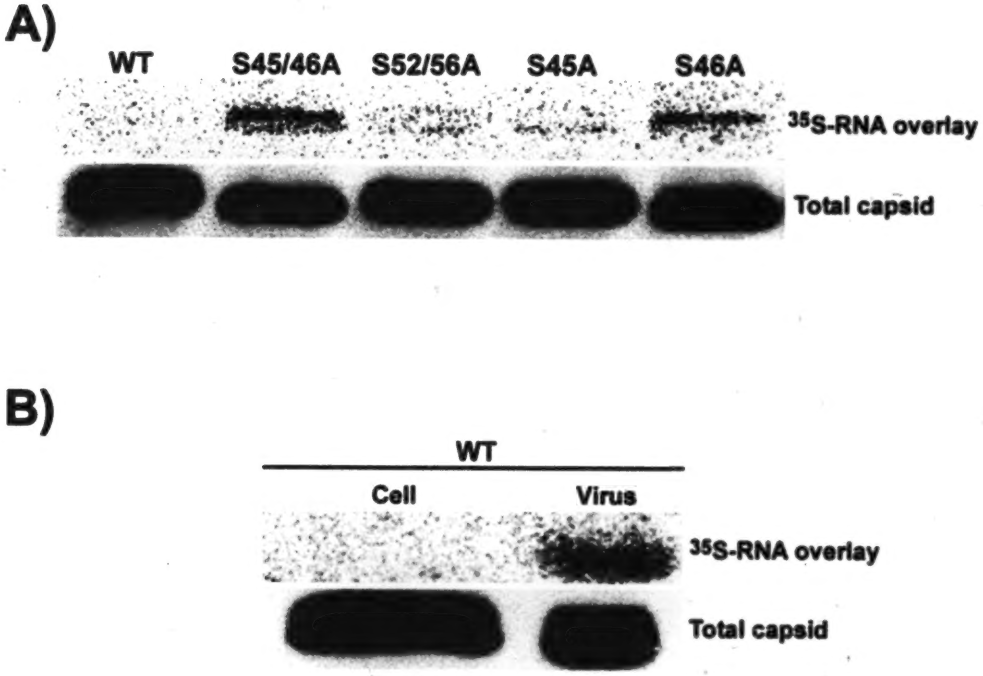

A)
WT
S45/46A
S52/56A
S45A
S46A
^{35}S-RNA overlay
Total capsid
B)
WT
Cell
Virus
^{35}S-RNA overlay
Total capsid

Figure 4.4. Phosphorylation of capsid negatively regulates RNA binding. A) Capsid proteins were isolated from transfected COS cells by immunoprecipitation using rabbit anti-capsid antibodies, separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with ^{35}S-labeled RV-specific RNA, washed and RNA-binding to capsid proteins was detected using a phosphorimager (upper panel). Relative capsid expression levels were assessed by stripping membranes and immunoblotting with a mouse monoclonal antibody to capsid (lower panel). B) Cell- and virion-associated capsids (virus) were isolated from cells infected with the M33 strain of RV. RNA binding to cell- and virion-associated capsids was determined as described for (A).

4.4B). In contrast, capsids isolated from lysates of infected cells did not bind detectable amounts of RNA in the *in vitro* RNA binding assay (Figure 4.4B).

The results described above indicate that virion-associated capsids differ from cellular capsids in some manner that affects their ability to bind RNA. To ascertain whether differences in phosphorylation could account for these observations, I tested the abilities of cell-associated capsids to interact with RNA after treatment with phosphatase. As expected, phosphatase-treatment of wild type, S52/56A and S45A capsids resulted in increased binding of viral RNA (Figure 4.5). However, the RNA-binding activities of the hypophosphorylated capsid mutants (S45/S46A and S46A) were not affected by phosphatase treatment. These results suggest that phosphorylation of serine 46 regulates capsid-RNA interaction. Together, the data are consistent with a scenario where capsid undergoes a dephosphorylation step prior to interacting with viral RNA, a process necessary for subsequent nucleocapsid formation.

4.5 Capsid undergoes dephosphorylation before or during packaging into virions

In order to directly show that capsid is dephosphorylated before or during packaging into virions, the phosphorylation levels of intracellular and virion-associated capsids were compared. Cells infected with the wild type M33 strain RV were grown in the presence of [^{32}P]-orthophosphate. Three days post-infection, intracellular and virion-associated capsids were immuno-affinity purified using a polyclonal antibody against capsid and a monoclonal antibody to E1 respectively. Since E1 is exposed on the virion surface, any capsids isolated using E1 antibodies are virion-associated. Samples were then subjected to SDS-PAGE and fluorography. In order to control for the variation in

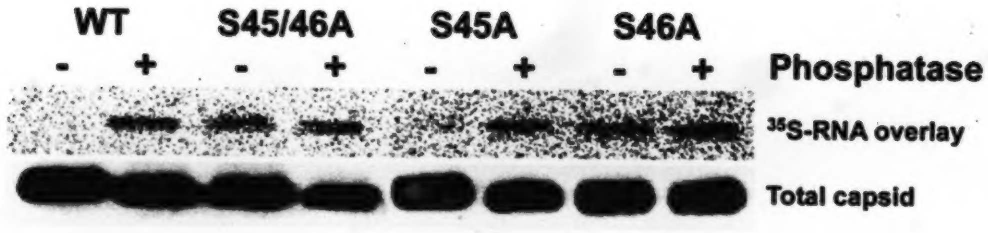

WT
S45/46A
S45A
S46A
- + - + - + - +
Phosphatase
35S-RNA overlay
Total capsid

Figure 4.5. Dephosphorylation of capsid increases its affinity for genomic RNA. Purified capsids were treated with or without calf intestinal phosphatase, separated by SDS-PAGE and then transferred to nitrocellulose membranes. Membranes were incubated with ^{35}S-labeled RV-specific RNA, washed and RNA-binding to capsid was detected with a phosphorimager (upper panel). Relative capsid expression levels were assessed by stripping membranes and immunoblotting with a mouse monoclonal antibody to capsid (lower panel).

protein levels of intracellular capsids versus virion-associated capsids, the phosphorylation level was normalized to the amount of total capsid within the same blot. Quantitation using a phosphorimager revealed that the virion-associated capsids contain ~40% of the radioactive-phosphate associated with the intracellular capsids (Figure 4.6). These results are consistent with a scenario in which capsid proteins undergo a dephosphorylation step before they are packaged into virions. Alternatively, it is possible that dephosphorylation could be happen after the step of virus assembly but prior to secretion. In addition, I cannot distinguish at this point if all capsid proteins undergo dephosphorylation to the same degree, or whether only a fraction of the capsid pool becomes completely dephosphorylated within a virion.

Based on sequence analysis, serine 46 is predicted to be a substrate for protein phosphatase 1A (PP1A). Indeed, PP1A and the general specificity phosphatase calf intestinal alkaline phosphatase, but not other phosphatases (PP1B, PP2A or PP2B), catalyzed removal of radiolabeled phosphate from the RV capsid in an *in vitro* dephosphorylation assay (Figure 4.7).

4.6 Virions containing hypophosphorylated capsid exhibit early replication defects

Both *in vitro* and *in vivo* results indicate that phosphorylation of capsid negatively regulates the capsid-RNA interaction. Dephosphorylation appears to be a molecular switch that allows capsid to interact with the genomic RNA, a process that is required for the formation of the nucleocapsid and subsequent virus budding. On the other hand, if phosphorylation of capsid can dynamically regulate its RNA binding activity, rephosphorylation of capsid may be expected to decrease its RNA binding activity in

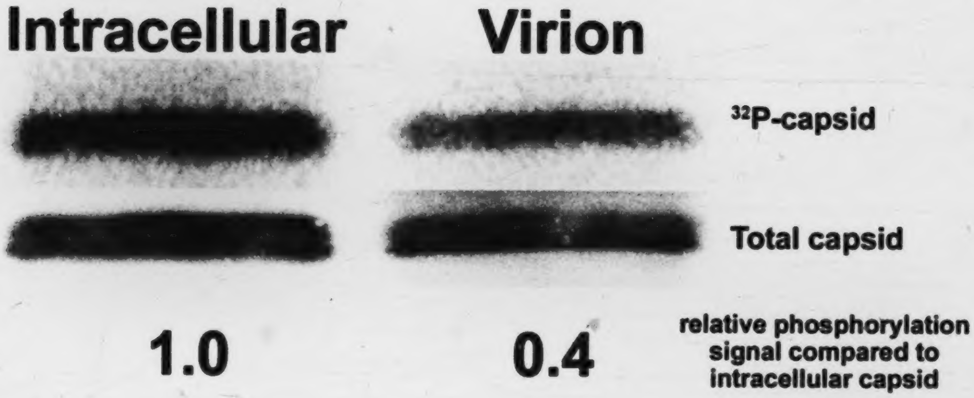

Intracellular
Virion
^{32}P-capsid
Total capsid
1.0
0.4
relative phosphorylation signal compared to intracellular capsid

Figure 4.6. Virion-associated capsids contain less phosphate than cell-associated capsids. Vero cells cultured in the presence of [^{32}P]-orthophosphate were infected with M33 RV (MOI = 10). Three days post-infection, intracellular and secreted virion-associated capsids were isolated by immunoprecipitation. Samples were subjected to SDS-PAGE and then blotted to nitrocellulose membranes. Phosphorylated capsid was detected with a phosphorimager. Relative capsid expression levels were assessed by stripping membranes and immunoblotting with a mouse monoclonal antibody to capsid (lower panel). The phosphorylation signals were normalized to the total capsid protein levels. The normalized phosphorylation level of intracellular capsid was set to 1.0. Values are the average of two independent experiments.

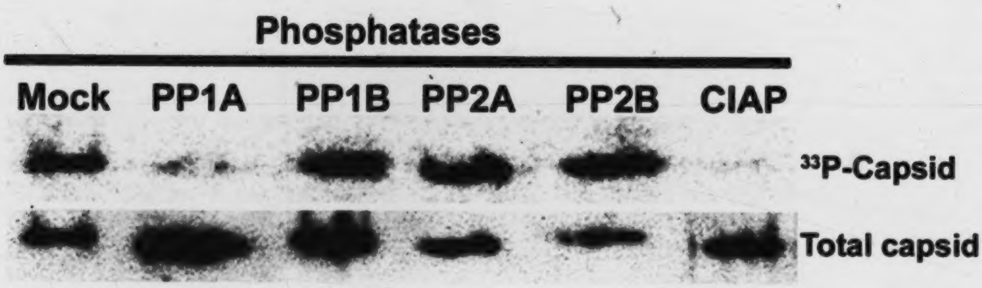

Phosphatases
Mock
PP1A
PP1B
PP2A
PP2B
CIAP
33P-Capsid
Total capsid

Figure 4.7. Capsid is dephosphorylated by protein phosphatase 1A (PP1A) *in vitro*. ^{33}P-labeled GST-capsid was incubated with or without PP1A, PP1B, PP2A, PP2B or calf intestinal phosphatase (CIAP) for 16 hours at 37 °C. Samples were then subjected to SDS-PAGE and transferred to PVDF membrane. The top panel shows ^{33}P-labeled GST-capsid as detected by a phosphorimager. The lower panel is an immunoblot with anti-GST antibody to show the total levels of GST-capsid protein in each lane. (Courtesy of Jason Everitt, University of Alberta)

order to facilitate nucleocapsid disassembly following endocytosis. I next hypothesized that phosphorylation of capsid is important to mediate efficient release of the viral genome during entry.

If this hypothesis has merit, the S46A capsid, as a result of its high RNA affinity, is expected to be less efficient in releasing virus genome during the virion disassembly stage. In turn, this would be expected to delay expression of viral proteins because the genomic RNA would not be transcribed as rapidly or as efficiently. To address this scenario, the expression profiles of viral proteins in cells infected with M33 and S46A mutant viruses were compared by western blotting using antibodies to the non-structural protein p150 and capsid. In accordance with my prediction, the viral proteins (p150 and capsid) were seen to accumulate earlier in lysates of cells infected by M33 (Figure 4.8 A and B). At high MOI (MOI=5), p150 in M33 infected cells was detectable by 16 hours post-infection and steadily accumulated until 36 hours post-infection. In contrast, the expression of p150 was not detected until 36 hours post-infection and the peak level of the protein was greatly reduced in S46A virus infected cells (Figure 4.8A upper panel). Accumulation of the capsid protein followed a similar trend as p150 (Figure 4.8A middle panel), in that capsids accumulated more rapidly and to higher levels in cells infected with M33 than with S46A virus. Low levels of capsid proteins were detected in both M33 and S46A virus infected cells at early time points. The appearance of capsid prior to accumulation of p150 may be explained by two reasons: 1) The anti-capsid antibodies are more sensitive than the anti-p150 antibodies; or 2) The low levels of capsid protein seen at early time points may represent residual nucleocapsids from incoming virions. The latter possibility is supported by the observation that capsid is undetectable at early time

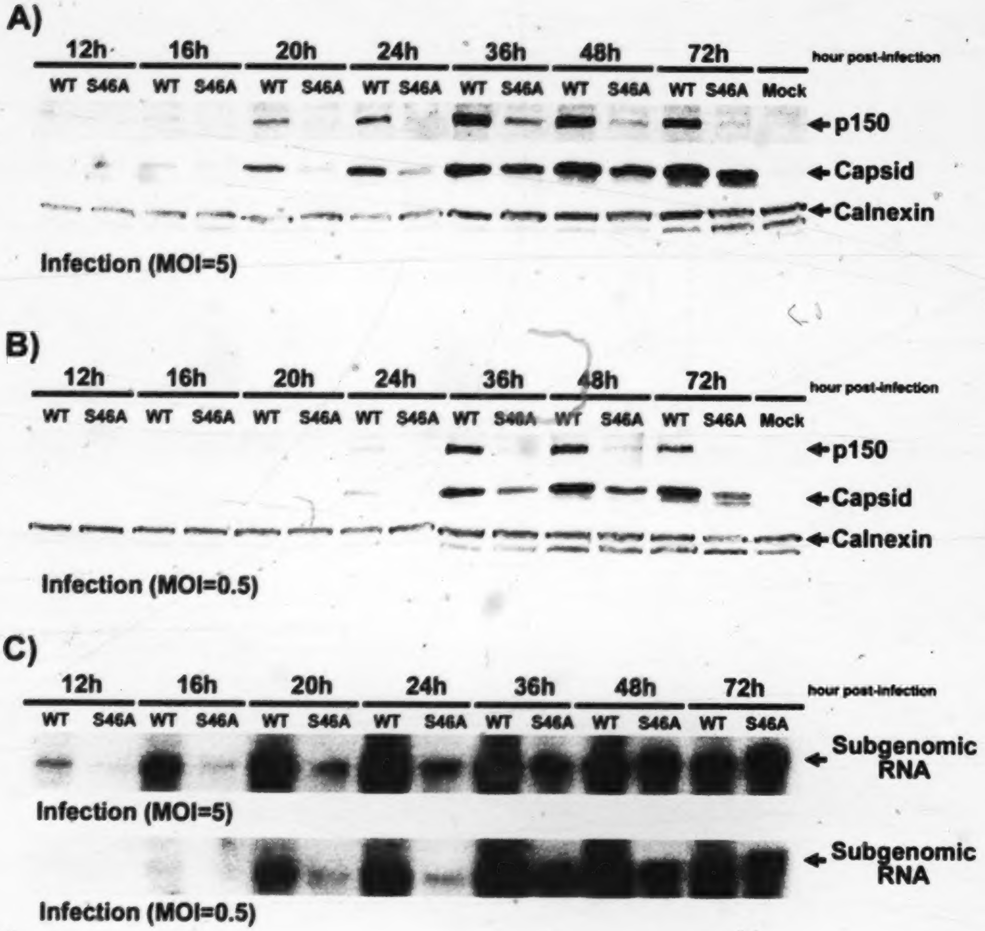

A)
12h
16h
20h
24h
36h
48h
72h
hour post-infection
WT S46A WT S46A WT S46A WT S46A WT S46A WT S46A WT S46A Mock
p150
Capsid
Calnexin
Infection (MOI=5)
B)
12h
16h
20h
24h
36h
48h
72h
hour post-infection
WT S46A WT S46A WT S46A WT S46A WT S46A WT S46A WT S46A Mock
p150
Capsid
Calnexin
Infection (MOI=0.5)
C)
12h
16h
20h
24h
36h
48h
72h
hour post-infection
WT S46A WT S46A WT S46A WT S46A WT S46A WT S46A WT S46A
Subgenomic RNA
Infection (MOI=5)
Subgenomic RNA
Infection (MOI=0.5)

Figure 4.8. Synthesis of virus proteins and RNA is delayed and reduced in Vero cells infected with the S46A virus. Vero cells were infected with either the M33 wild type or the S46A strains at MOI=5 (A, C top panel) and MOI=0.5 (B, C low panel). At regular intervals after infection, cell lysates were prepared and subjected to SDS-PAGE and immunoblotting with antibodies to p150 and capsid (A, B). The membranes were also probed with anti-calnexin antibodies as a control for protein loading (A, B). In addition, total RNAs were isolated from infected cells. The levels of RV subgenomic RNA were determined by northern blotting (C). (Courtesy of Dr. Wen-Pin Tzeng, Georgia State University, U.S.A)

points when lower MOIs were used (Figure 4.8B) or when the genome was delivered by transfection (Figure 4.9A).

Next, the kinetics of subgenomic RNA accumulation was monitored by northern-blot analysis. Similar to the western blot results, infection by the wild type virus resulted in a more rapid buildup of the subgenomic RNA compared to the S46A mutant virus (Figure 4.8C). This suggests that the S46A mutant virus is delayed and/or impaired in both the transcription of the subgenomic RNA and in the translation of viral proteins (p150 and capsid), all of which are consistent with a defect in nucleocapsid disassembly. This effect is independent of the amount of virus present during infection as evidenced by the fact that similar results were obtained when the experiments were performed at both high and low MOI (Figure 4.8).

Based on recent reports establishing the role of capsid in controlling viral transcription (Chen and Icenogle, 2004; Tzeng and Frey, 2003), it has been suggested that proper phosphorylation of capsid is required for enhancing virus replication. Therefore, it was essential to rule out the possibility that the delay in expression of viral proteins and RNAs was simply the result of different rates of viral replication in wild type virus versus the S46A mutant virus. In order to test this possibility, a similar experiment to that described above was performed except that the viral genome was delivered by transfecting Vero cells with *in vitro* transcribed capped viral RNA. This method bypassed the step of virus uncoating and therefore removed capsid-RNA interaction as a factor. Equal amounts of viral transcripts encoding either the M33 or the S46A mutant virus were transfected into Vero cells. Expression of virus proteins and subgenomic RNA in transfected cells was monitored as described above. Interestingly, regardless of whether

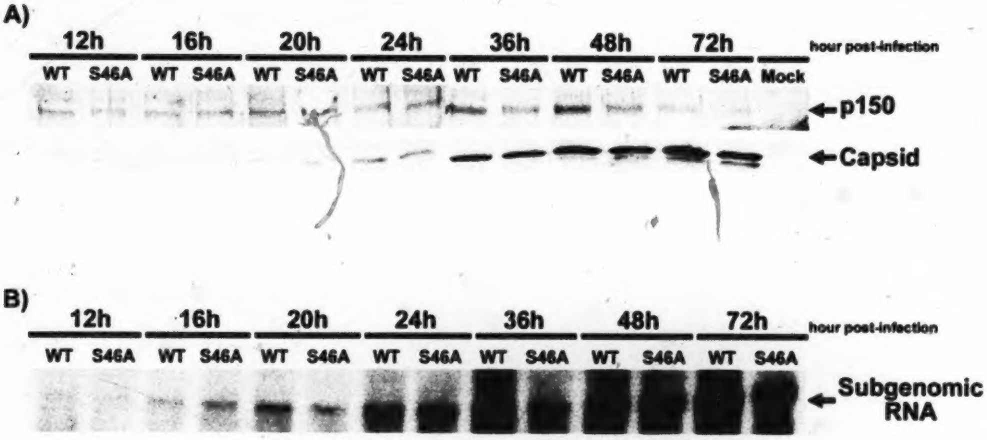

A)
12h
16h
20h
24h
36h
48h
72h
hour post-infection
WT S46A WT S46A WT S46A WT S46A WT S46A WT S46A WT S46A Mock
p150
Capsid
B)
12h
16h
20h
24h
36h
48h
72h
hour post-infection
WT S46A WT S46A WT S46A WT S46A WT S46A WT S46A WT S46A
Subgenomic RNA

Figure 4.9. Similar kinetics for capsid, p150 and subgenomic RNA accumulation were observed in cells transfected with M33 (WT)- and S46A-specific RNAs. To bypass the uncoating step, virus genomes were delivered into Vero cells by transfection. At various time points, the levels of A) virus proteins (capsid and p150) and B) subgenomic RNA were determined as described in Figure 16. (Courtesy of Dr. Wen-Pin Tzeng, Georgia State University, U.S.A.)

cells were transfected with either M33 or S46A RNAs, both capsid and p150 accumulated with similar kinetics (Figure 4.9A). In addition, the levels of subgenomic RNA in the transfected cells were similar at early and middle time points (12 to 24 hours) (Figure 4.9B). Together, these data indicate that mutation of the capsid gene does not directly affect transcription and translation of the virus genome. Moreover, it appears that the replication defects associated with the S46A mutant virus are due to a delay in virus uncoating or nucleocapsid disassembly, which results in delayed and/or reduced viral RNA and protein expression.

4.7 Virion-associated capsid does not change its phosphorylation state after entering the host cell

The fact that early replication defects are observed with the S46A virus, is consistent with the notion that capsid phosphorylation promotes disassembly of the nucleocapsid. A simple mechanistic explanation is that the incoming virion-associated capsids are phosphorylated in order to lower their RNA binding affinities, thereby facilitating release of the viral genome. The fact that the S46A capsid cannot be properly phosphorylated may account for the delay in expression of viral proteins and RNAs in infected cells. In order to directly show a role for capsid phosphorylation in virus uncoating, I investigated whether or not the phosphorylation state of capsid changes during the initial stage of viral infection, i.e. do the incoming capsids become phosphorylated? Vero cells infected with M33 RV (MOI = 10) were cultured in the presence of [^{32}P]-orthophosphate and capsids were immuno-purified from cell lysates at regular intervals. The immunopurified capsids were then separated by SDS-PAGE and processed for fluorography.

De novo synthesis of capsid protein occurs between 12 and 20 hours post infection. This process was evidenced by the sharp increase in capsid levels at 20 hours post-infection (Figure 4.10A, lane 6 and 7, lower panel). Phosphorylation of the nascent capsids was readily detected at the same time points (Figure 4.10A, lanes 6 and 7, upper panel). In contrast, capsids derived from incoming viruses did not incorporate detectable amounts of ^{32}P (Figure 4.10A, lane 1 to 5). This indicates that these capsid proteins do not undergo phosphorylation during the uncoating of virions. In order to eliminate doubt that the lack of phosphorylation of incoming capsid is due to the relatively low levels of this protein during early infection, the experiment was repeated by scaling up the amount of infected cells in an effort to collect enough capsid protein from the incoming virions. In addition, the samples from the pool of phosphorylated capsids during the later stages of infection were diluted to demonstrate that the signal from the phosphate can be detected among relatively small quantities of phosphorylated capsid. In figure 4.10B (lanes 3 and 4), the levels of capsid were barely detectable by western blotting, but the radioactive-phosphate labeled capsid was clearly visible. On the other hand, despite the high level of capsid protein collected four hours post-infection, the phosphorylation signal was undetectable (Figure 4.10B, lane 2). These results indicate that the capsid protein from incoming virions does not undergo phosphorylation after nucleocapsid disassembly and suggests that rephosphorylation is not required for nucleocapsid disassembly.

4.8 Capsid has multiple phosphorylation sites

Most of this study has been centered on the characterization of the hypophosphorylated capsid mutant CapS46A. I was of course also interested in examining how constitutive phosphorylation of capsid would affect virus replication. As

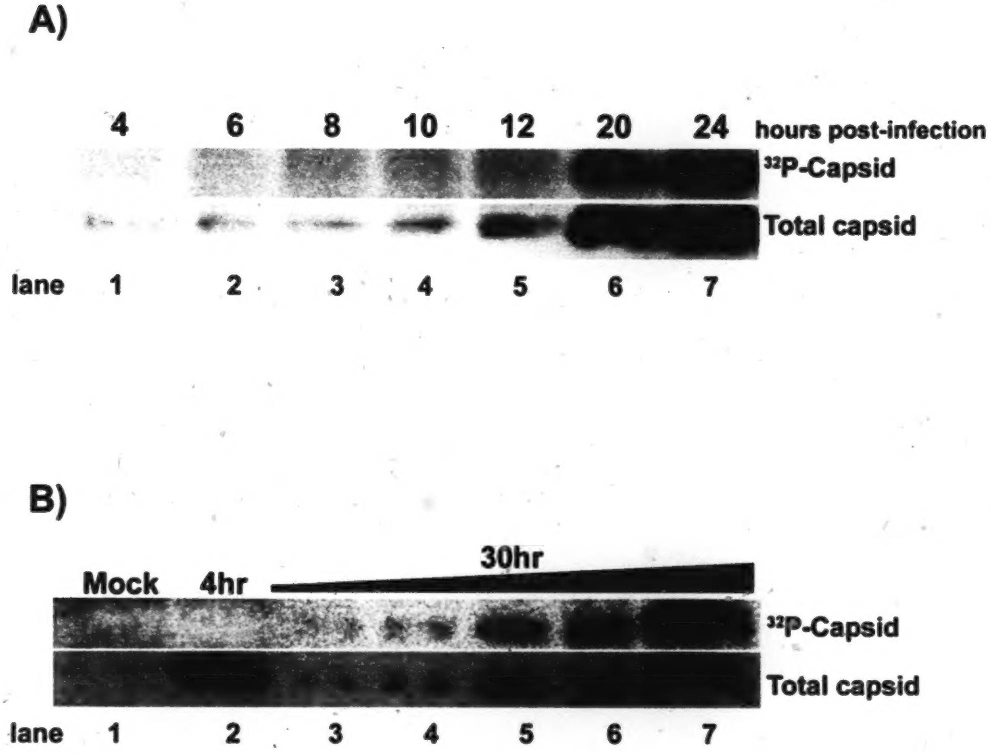
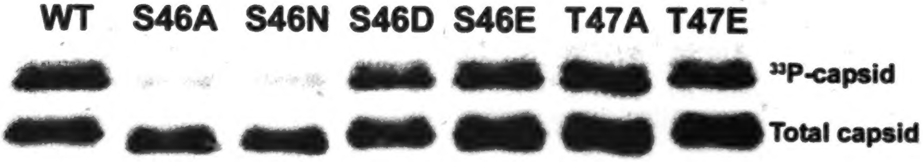


Figure 4.10. Incoming capsids do not undergo rephosphorylation. Vero cells were infected with M33 RV (MOI=10). At regular intervals post-infection, intracellular capsids were isolated by immunoprecipitation. Samples were then subjected to SDS-PAGE and fluorography. Relative capsid levels were determined by probing membranes with the mouse monoclonal antibodies to capsid followed by ECL detection (lower panels). A) Infection was carried out in media containing [^{32}P]-orthophosphate. The time-course of capsid accumulation and phosphorylation during infection is shown. B) In order to collect relatively large amounts of capsid protein from incoming virions, twice the amount of infected cells were used relative to (A). RV-infected cells (either 4 hours post-infection or 30 hours post-infection) were labeled with [^{32}P]-orthophosphate for three hours. Intracellular capsids were then isolated by immunoprecipitation. Increasing amounts of capsid proteins from the sample collected at 30 hours post-infection was loaded to illustrate that the phosphate signal can be detected among relative small amounts of phosphorylated capsid (lane 3 and 4).

serine 46 is clearly the key amino acid residue that regulates capsid phosphorylation (Figure 4.2), I elected to construct capsid mutants that would mimic constitutive phosphorylation at serine 46. Initially, serine 46 was replaced with the acidic amino acid residues aspartate and glutamate to create CapS46D and CapS46E respectively. The size and charge of the carboxyl side chains of aspartate and glutamate are able to mimic phosphorylated serine or threonine residues (Kock et al., 2003; Wu et al., 2002; Yueh and Goff, 2003). I first tested the phosphorylation state of these capsid constructs by *in vivo* labeling with [^{33}P]-orthophosphate. Surprisingly, substitution of acidic amino acid residues at position 46 resulted in capsid proteins that appear to be phosphorylated to a similar extent as wild type capsid (Figure 4.11A), and indicate that capsid is phosphorylated at more than one site. The slightly lower levels of phosphate in CapS46D and CapS46E are consistent with the scenario that serine 46 is one of the major phosphorylated residues (Figure 4.11A). While substitution of acidic residues at position 46 can restore capsid phosphorylation, changing serine 46 to asparagine (CapS46N) effectively blocked capsid phosphorylation. Therefore, the negative charge on aspartate or glutamate is sufficient to mimic the phosphate group attached to serine 46 such that progressive downstream phosphorylation occurs in capsid. A capsid mutant in which serine residues 45, 46, 48, 52 and threonine 47 (CapP5D) within the RNA binding site were replaced by aspartate residues, was found to be minimally phosphorylated similar to CapS46A (Figure 4.11B). Based on the assumption that aspartate at position 46 can induce downstream phosphorylation, the lack of phosphorylation signal exhibited by CapP5D suggests that most if not all of the major phosphorylated amino acid residues of capsid reside within the RNA binding site. Together, these results suggest that

A)


WT
S46A
S46N
S46D
S46E
T47A
T47E
^{33}P-capsid
Total capsid

B)

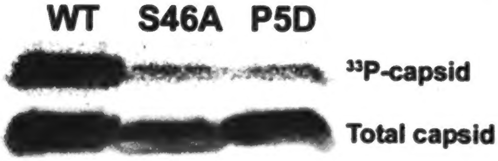

WT
S46A
P5D
^{33}P-capsid
Total capsid

Figure 4.11. Substitution of acidic amino acid residues at position 46 induces progressive phosphorylation of capsid. WT or mutant capsid constructs were transfected into COS cells and 24 hours post-transfection, cells were incubated with media containing [^{33}P]-orthophosphate for 12 hours prior to lysis. Samples were subjected to radioimmunoprecipitation with rabbit anti-capsid antibodies, SDS-PAGE, transfer to PVDF membrane and fluorography (upper panels). Relative expression levels of capsid proteins were determined by probing the same membranes with a monoclonal antibody to capsid followed by detection using ECL (lower panels). Experimental conditions were identical for (A) and (B).

phosphorylation of serine 46 allows one or more of these residues (serine 45, 48, 52, and threonine 47) to be phosphorylated.

Initially, there were seven potential phosphorylation residues identified within the RNA binding site (Table 4.1). However, after examining an extensive series of capsid mutants, I have narrowed down the likeliest candidates for phosphorylation to four serine/threonine residues within this region. I conclude that serine 34 and 45 are not phosphorylated based on the observation that mutation of these residues to alanine (CapS34A and CapS45A), did not affect the overall level of capsid phosphorylation. In addition, based on the observation that CapP6D and CapP5D exhibited the same levels of phosphorylation, it is likely that serine 56 is not phosphorylated (Table 4.1). That leaves four potential phosphorylation sites: serine 46, 48, 52 and threonine 47 within the RNA binding site. These are the best sites to focus on in order to further identify specific phosphorylated residues in the RNA binding site.

4.9 RNA binding activity of capsid is not directly regulated by phosphorylation of serine 46

The replacement of serine 46 with acidic amino acid residues (aspartate and glutamate) was found to effectively mimic phosphoserine and induce progressive downstream phosphorylation of capsid. Next, I investigated whether these residues could mimic the effect of phosphorylation in inhibiting the interaction between capsid and genomic RNA. Based on the previous finding that phosphatase treatment can enhance the RNA binding of capsid (Figure 4.5), I hypothesized that the phosphorylated serine/threonine residue(s) within the RNA binding site inhibits the capsid-RNA interaction. If acidic amino acid residues can mimic the effect of phosphorylation, the substitution of acidic residues is expected to block phosphorylation of capsid and inhibit capsid-RNA interaction.

Interestingly, the single aspartate substitution mutant CapS46D had low RNA binding activity (Figure 4.12). However, the RNA binding activity of this protein was enhanced after phosphatase treatment indicating that dephosphorylation of another residue(s) is essential for this process. Since this protein is heavily phosphorylated (Figure 4.11A), these data indicate that other phosphorylated amino acid residues in capsid inhibit the interaction with genomic RNA, i.e., the positive charge at position 46 of CapS46D allows downstream phosphorylation of other amino acid residues that in turn prevent RNA binding. The reason that CapS46D exhibits low RNA affinity is presumably due to phosphorylation of unidentified residue(s) downstream of serine 46. Therefore, phosphorylation at serine 46 is critical for inducing phosphorylation of other amino acid residues that directly regulate RNA binding activity. Moreover, blocking phosphorylation of serine 46 without affecting the phosphorylation of other residues, as in the case of CapS46D, does not enhance capsid-RNA interaction.

In the search for which residues are essential in regulating the RNA binding activity of capsid, I looked for mutants that bound RNA efficiently when S46 was phosphorylated (Table 4.1). CapA3 and CapA4-2 are the two constructs that fit these criteria. These constructs, despite being phosphorylated, bind RNA efficiently (Table 4.1). This suggests that the overlapping mutations in these constructs threonine 47, serines 48 and 52 could be the essential residues that regulate the capsid-RNA interaction. I have further narrowed down the residue that is critical for regulating capsid-RNA interaction by examining other mutants. The mutant CapP3D, in which serines 45,

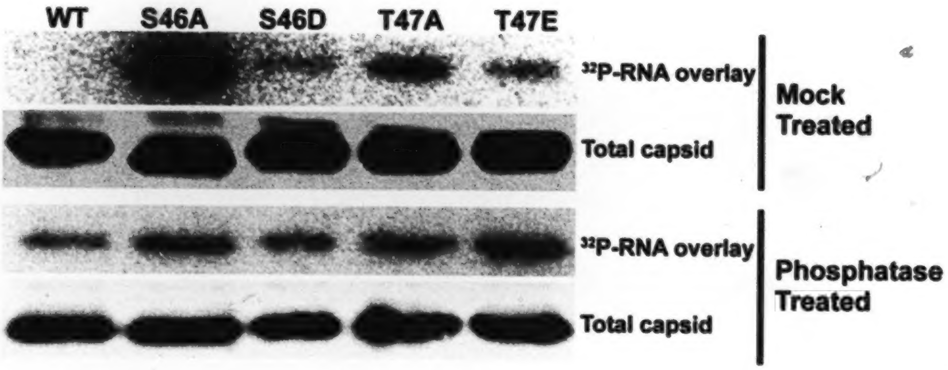

WT
S46A
S46D
T47A
T47E
32P-RNA overlay
Total capsid
Mock Treated
32P-RNA overlay
Total capsid
Phosphatase Treated

Figure 4.12. The effect of acidic amino acid substitutions at positions 46 and 47 on capsid-RNA interactions. Capsid proteins (treated with or without calf intestinal phosphatase) were isolated from transfected COS cells by immunoprecipitation, separated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated with ^{32}P-labeled RV-specific RNA, washed and RNA-binding to capsid proteins was detected using a phosphorimager (upper panel). Relative capsid expression levels were determined by stripping the membranes and immunoblotting with a monoclonal antibody to capsid (lower panel).

46 and threonine 47 are replaced with aspartate residues, binds RNA efficiently and is heavily phosphorylated (Table 4.1). The ability of P3D to bind RNA suggests that serines 48 and 52 are not critical for capsid-RNA interaction, because these residues are available for phosphorylation in CapP3D. I hypothesize that threonine 47 is more important than serines 48 and 52 in regulating capsid-RNA interaction.

To directly assess the importance of threonine 47 in capsid phosphorylation and RNA binding, I constructed a capsid mutant in which threonine 47 was changed to alanine. The resulting construct CapT47A exhibited higher RNA binding activity compared to the wild type capsid or CapS46D (Figure 4.12), despite being heavily phosphorylated (Figure 4.11A). Also, phosphatase treatment did not increase RNA binding activity of T47A (Figure 4.12). This indicates that blocking phosphorylation at amino acid residue 47 is sufficient to enhance the RNA binding activity of capsid without affecting the overall phosphorylation state of capsid.

In an effort to construct a recombinant capsid that mimics constitutive phosphorylation at threonine 47 of capsid, I substituted threonine 47 with glutamate. The resulting mutant CapT47E behaved similarly to CapT47A in that it was heavily phosphorylated (Figure 4.11A). However, CapT47E consistently showed a lower RNA binding activity than CapT47A or other hypophosphorylated capsids such as CapS46A (Figure 4.12B, upper panel). Although it appears that CapT47E exhibits slightly higher RNA binding activity than wild type capsid (Figure 4.12B, upper panel) subsequent experiments showed that CapT47E behaved similarly to the wild type capsid in that it does not bind RNA efficiently (Table 4.1). Surprisingly, the RNA binding activity of CapT47E was restored after phosphatase treatment (Figure 4.12B, lower panel). At this point, it seems rather contradictory that the introduction of glutamate at threonine 47 mimics phosphorylation to inhibit RNA-binding, but that phosphatase treatment of T47E restores the capsid-RNA interaction. One logical explanation is that substitution of glutamate at amino acid residue 47 induces phosphorylation of other amino acid residues that are not phosphorylated in CapT47A. Further work is required to define the role of phosphorylation at threonine 47 in capsid-RNA interaction.

4.10 Summary

In this section, I have examined the role of capsid phosphorylation in virus replication. First, it was determined that the major phosphorylated residues are located within the RNA binding site of capsid. Using site-directed mutagenesis and deductive reasoning, I conclude that there are a maximum of four phosphorylated amino acid residues in the capsid: serines 46, 48, 52 and threonine 47. Of these four residues, serine 46 is the key residue that must be phosphorylated before downstream phosphorylation can occur. Downstream phosphorylation of amino acid residues requires a negative charge at position 46 as evidenced by the fact that substitution of aspartate or glutamate, but not asparagine or alanine, at this position allows phosphorylation of capsid.

Subsequently, I showed that regulated phosphorylation of capsid is important for virus replication. Recombinant viruses that encode hypophosphorylated capsids (S46A or S45/46A) reached peak titers that were as much as 60 fold lower than the wild type M33 strain virus. In addition, these mutant viruses exhibited less cytopathogenicity.

Capsid phosphorylation is important for virus replication because this post-translational modification modulates the RNA binding activity of capsid. Specifically, phosphorylation of capsid negatively regulates the capsid-RNA interaction. Based on biochemical studies, phosphorylation at threonine 47 is particularly important. The phosphorylation status of this amino acid residue appears to directly influence the RNA binding activity of capsid. During the early stages of virus assembly, capsid is heavily phosphorylated, a situation that would be expected to prevent interaction with the genomic RNA before structural proteins are targeted to the budding site. Presumably, the phosphorylation of capsid may also prevent non-specific binding to cellular RNAs. At a later stage of the virus life cycle, capsid is dephosphorylated, possibly by PP1A at the Golgi complex, thus allowing interaction with the genomic RNA, a step necessary for the formation of the nucleocapsid. Together, the phosphorylation of capsid behaves as a molecular switch regulating the capsid-RNA interaction, and ultimately nucleocapsid formation. Consistent with this idea, virion-associated capsids are not as heavily phosphorylated as cell-associated capsids.

Viruses that harbor hypophosphorylated capsid proteins, have longer latent periods, which is consistent with a defect in nucleocapsid disassembly. However, I did not find evidence to suggest that rephosphorylation of the incoming capsid proteins is required for this step. Based on the fact that S46A virions exhibit a defect at the step of uncoating, phosphorylation of capsid is likely serves an as yet unknown function during virus entry.

Chapter 5 . Discussion

5.1 Study of RV

Togaviruses have served as classical models for the study of virus assembly, replication and host cell entry (Helenius and Marsh, 1982; Simons et al., 1982). Within the togaviruses, the well-studied alphaviruses have served as a useful paradigm for understanding many aspects of RV biology. However, as more is learned about these genera of viruses, the more it becomes apparent that they possess many significant differences. One of theses differences is the process of nucleocapsid assembly, which for RV is a tightly regulated event and occurs in association with Golgi membranes. During RV assembly, the main function of the capsid is to package the genomic RNA and form the nucleocapsid core. In order to understand the process of nucleocapsid formation, I elected to study how capsid functions during RV replication. For these studies, capsid was divided into three distinct regions: I) the amino-basic region that includes the RNA binding site for packaging genomic RNA; II) the central region; and III) the carboxyl-hydrophobic region that also functions as the E2 SP (Figure 1.5). In this study, I have focused on two of the functional regions of capsid. Initially, I examined the role of the E2 SP, the carboxyl-hydrophobic region of capsid, in virus assembly. Second, I determined the major phosphorylated amino acid residues in capsid and characterized the role of phosphorylation in this protein during virus replication. Since the majority of the phosphorylated amino acid residues are located within the RNA binding site of capsid, the role of this post-tanslational modification in regulation of the capsid-RNA interaction and its subsequent effects in virus replication were examined.

5.2 The role of E2 signal peptide

The retention of a signal peptide at the carboxyl end of RV capsid is unique among related RNA viruses (Hobman and Gillam, 1989; Suomalainen et al., 1990). Viruses such as alphaviruses and members of the family *Flaviviridae* (e.g., West Nile virus and hepatitis C virus) have evolved different mechanisms to remove the signal peptides of the following glycoproteins from the mature capsid proteins. In alphaviruses, capsid proteins process a protease domain that catalyzes the structural polyprotein cleavage to separate capsid from the following glycoproteins (Melancon and Garoff, 1987). The cleavage takes place upstream of the signal peptide for the first glycoprotein E2. As a result, alphavirus capsids are soluble proteins and formation of the nucleocapsid takes place in the cytoplasm in the absence of membranes. Within the family *Flaviviridae*, which includes three genera Flaviviruses, Pestiviruses and Hepaciviruses, two endoproteolytic cleavages are required to remove the glycoprotein signal peptide from the core protein (the terms capsid and core proteins are used interchangeably for *Flaviviridae*, (Lindenbach, 2001)). The first cleavage is similar to that which occurs for RV capsid. Specifically, the host signal peptidase cleaves after the signal peptide of the following glycoprotein to remove the core protein from the polyprotein. This is followed by a step unique to flaviviruses, in which a virus-encoded protease cleaves upstream of the signal peptide, thus removing the hydrophobic domain from the core protein (Amberg et al., 1994; Lobigs, 1993; Yamshchikov and Compans, 1995). It was assumed that this mechanism to process the core protein is conserved within the family *Flaviviridae*, but it was recently discovered that hepatitis C virus utilizes the host presenilin-type signal peptide peptidase for removal of the signal peptide from core (Lemberg and Martoglio, 2002; McLauchlan et al., 2002; Weihofen et al., 2002). This recently identified host signal peptide peptidase catalyzes an intramembrane cleavage to remove a large portion of the signal peptide from the carboxyl terminus of hepatitis C virus core protein (McLauchlan et al., 2002). Removal of the signal peptide is essential for retaining hepatitis C virus core at the ER, where virus assembly occurs (Okamoto et al., 2004). These examples of different mechanisms utilized by different RNA viruses to remove the hydrophobic domains from capsid proteins may reflect the distinct assembly pathways of these different viruses. The retention of the E2 SP at the carboxyl terminus of RV capsid may be important for the membrane-associated assembly of the nucleocapsid, which occurs at the Golgi complex.

Experimental evidence presented here indicates that the RV E2 SP is multifunctional. It first serves to initiate translocation of E2 into the ER and then subsequently functions as a membrane anchor for capsid (Suomalainen et al., 1990). Suomalainen *et al.* hypothesized that this may provide the mechanism to account for the membrane-dependent assembly of RV nucleocapsids. My results are certainly consistent with this notion and, in addition, clearly show that the E2 SP has an additional function at an early step in the virus assembly pathway. Specifically, this domain is necessary for E2/E1-dependent targeting of capsid to the juxtanuclear region where virus budding occurs. Previous studies by our lab demonstrated that the folding and transport of E2/E1 heterodimers from the ER to the Golgi is a highly coordinated process, and that maturation of E1 in the ER is the rate-limiting step for transport (Garbutt et al., 1999). Presumably, E2 and E1 are transported from the ER to the Golgi in a COPII/COPI-dependent manner similar to other viral and cellular membrane proteins. However, until this study, nothing was known about how the capsid protein reaches the Golgi complex, the site of virus assembly. The indirect immunofluorescence data presented here clearly show that the E2 SP is required for transport of capsid to the juxtanuclear region (Figure 3.5). It is tempting to speculate that an interaction between the E2 SP and one or more of the three other membrane-spanning domains on E2 or E1 (i.e. E2 TM, E1 SP or E1 TM) directs capsid into the same ER-derived transport vesicles as the glycoproteins. This process would serve to coordinate the transport of capsid to the Golgi complex with that of E2 and E1 (Figure 5.1). In agreement with this idea, replacement of the E2 TM with the VSV G TM not only disrupted the targeting of E2 to the Golgi complex, but also affected the targeting of capsid to the Golgi region (Garbutt et al., 1999). However, replacement of the E1 TM with the VSV G TM did not affect the targeting of capsid (Garbutt et al., 1999). This implies that interaction between the E2 and capsid transmembrane domains is particularly important for proper targeting of capsid to the Golgi complex.

The putative lateral interactions between the E2 SP and the E2 TM may not be sufficient for mediating capsid and glycoprotein interaction. For example, non-conservative substitutions of the arginine residues within the cytoplasmic domain of E2 resulted in abnormal localization of both E2 and capsid (Garbutt et al., 1999). It has been proposed that the charged residues within the cytoplasmic domain of E2 are important to mediate electrostatic interactions with the capsid (Garbutt et al., 1999). If this is the case, it would seem that both the hydrophobic interactions between the E2 SP of capsid and the E2 TM and electrostatic interactions between the cytoplasmic domain of E2 and capsid are required to coordinately mediate stable binding of capsid and E2.

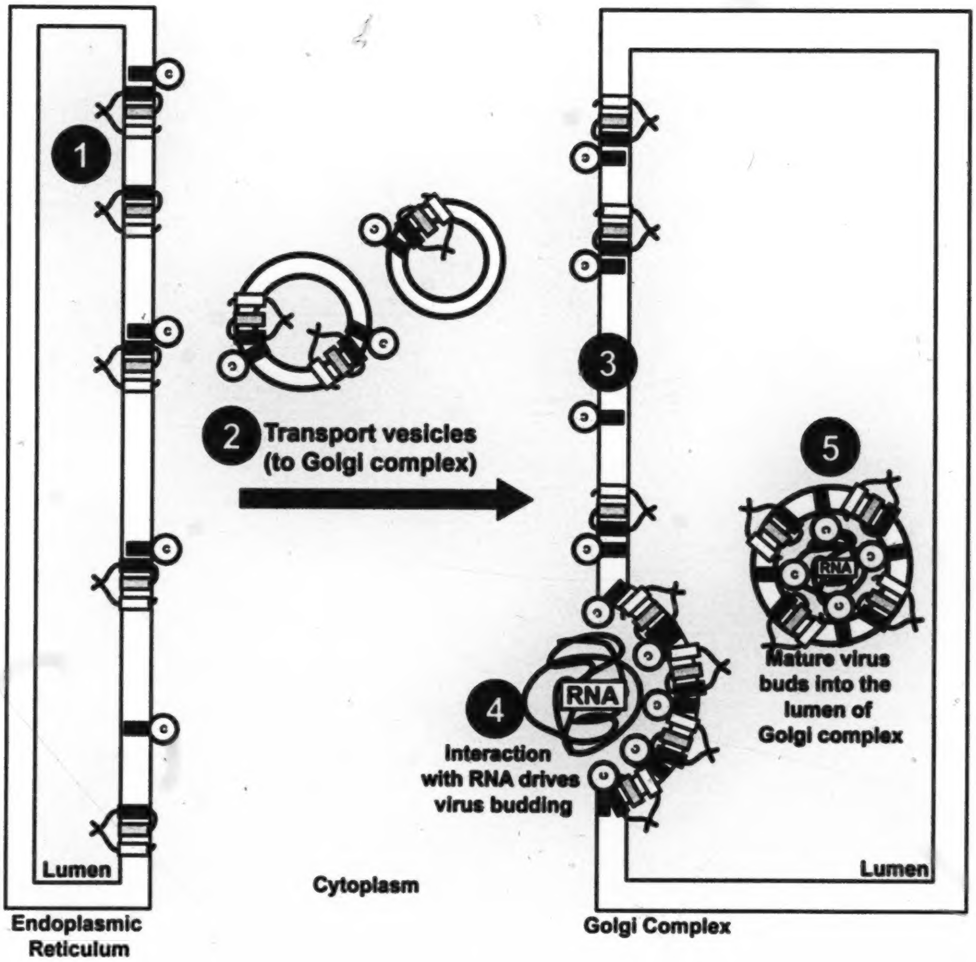

1
2 Transport vesicles (to Golgi complex)
3
4 Interaction with RNA drives virus budding
5 Mature virus buds into the lumen of Golgi complex
RNA
RNA
Lumen
Cytoplasm
Lumen
Endoplasmic Reticulum
Golgi Complex

Figure 5.1. Model to illustrate the role of E2 SP during virus assembly. 1) Structural proteins (capsid, E2 and E1) are synthesized at the ER. 2) Interaction between capsid and the glycoproteins is essential for proper targeting of capsid to the Golgi complex. This interaction is mediated via the lateral interaction between E2 SP at the carboxyl-terminus of capsid and the E2 TM. These interactions may be required for capsid to be transported in the same transport vesicles as the glycoproteins. 3) The E2 TM acts as a Golgi retention signal, which also retains capsid and E1 in the Golgi complex. 4) The lateral interaction between transmembrane domains of structural proteins could also serve to augment the process of virus budding. 5) Virions bud into the Golgi lumen to complete the virus assembly process.

Since E2 has a retention signal that functions to retain the glycoprotein heterodimer at the Golgi complex (Hobman et al., 1995), capsid may be prevented from traveling beyond the virus assembly site by virtue of its stable association with E2. Furthermore, interactions between the E2 SP and the transmembrane domains of the glycoproteins in the Golgi membranes, may augment assembly of the virus (Figure 5.1). It will be of interest to determine if the E2 SP has a role in initiating virus budding. As nucleocapsid formation and virus budding are coordinated events, it is conceivable that formation of nucleocapsids, in conjunction with the capsid-glycoproteins interaction, may provide the necessary force to drive virus budding into the lumen of the Golgi complex (Figure 5.1, see section 5.3).

While the advantage of retaining the E2 SP at the carboxyl terminus would be to coordinate the transport of capsid and glycoprotein to the virus budding site, the presence of this hydrophobic region may be energetically unfavorable for targeting capsid to mitochondria via passive diffusion. Indeed, the mechanism by which capsid is targeted to mitochondria is not yet understood. During RV infection, perinuclear clustering of organelles such as the ER, the Golgi complex and mitochondria have been reported (Lee et al., 1996; Risco et al., 2003). One possibility is that ER-anchored capsid interacts with mitochondria. Electron dense plaques have been observed between the outer membranes of ER and mitochondria (Lee et al., 1996). The close association of these organelles may facilitate transport of the protein between these organelles. In fact, expression of capsid alone is sufficient to induce electron plaques between two adjacent mitochondria. These plaques resemble those that are observed in RV infected cells (Beatch, 2004). Alternatively, interaction with host proteins may be able to mask the hydrophobic region of capsid to allow the transport of this protein to the mitochondria. Previously, capsid was thought to require binding to newly synthesized p32 for targeting to the mitochondria (Beatch and Hobman, 2000). However, it was later shown that capsid association with this organelle is independent of p32 (Beatch, 2004). Moreover, preliminary data in our lab suggest that the E2 signal peptide is not required for targeting of capsid to mitochondria. Rather, a putative amphipathic helix was found to be a part of mitochondria targeting signal in capsid (Everitt, unpublished result). Similar results were published for the hepatitis C virus core protein (Schwer et al., 2004). This suggests that capsid/core proteins of RV and hepatitis C virus may share a similar mechanism for targeting to the mitochondria. In addition, cleavage of the glycoprotein signal peptide at the carboxyl terminus of hepatitis C virus core protein is essential for proper targeting of the protein (McLauchlan et al., 2002; Okamoto et al., 2004). It would be of interest to determine if a similar process happens to RV capsid during infection. It is possible that the pool of capsid destined for mitochondria is proteolytically processed to remove the E2 signal peptide. Isolation and characterization of mitochondria associated RV capsid could address this possibility.

5.3 The role of capsid phosphorylation

Phosphorylation is one of many ways to modulate the functions of virus proteins during replication. Numerous studies indicate that phosphorylation of capsid proteins is essential for virus infection (Cartier et al., 1999; Chapdelaine et al., 2002; Ivanov et al., 2001; Kock et al., 2003; Lan et al., 1999; Leclerc et al., 1999; Maroto et al., 2000; Mohandas and Dales, 1991; Siddell et al., 1981; Yu and Summers, 1994a). While in many cases the specific roles of capsid phosphorylation have yet to be studied, this post-translational modification is known to affect various functions such as genome packaging (Cuesta et al., 2000; Gazina et al., 2000; Ivanov et al., 2001; Kann and Gerlich, 1994; Lan et al., 1999) and subcellular localization (Kann et al., 1999; Kock et al., 2003; Liao and Ou, 1995; Lu and Ou, 2002; Rabe et al., 2003). Although there are examples of phosphorylation occurring in togaviruses capsids (Marr et al., 1994; Waite et al., 1974), surprisingly, nothing is known about how this post-tanslational modification regulates capsid function. In this study, I have identified several phosphorylated amino acid residues that are critical for regulating RV capsid phosphorylation and virus replication.

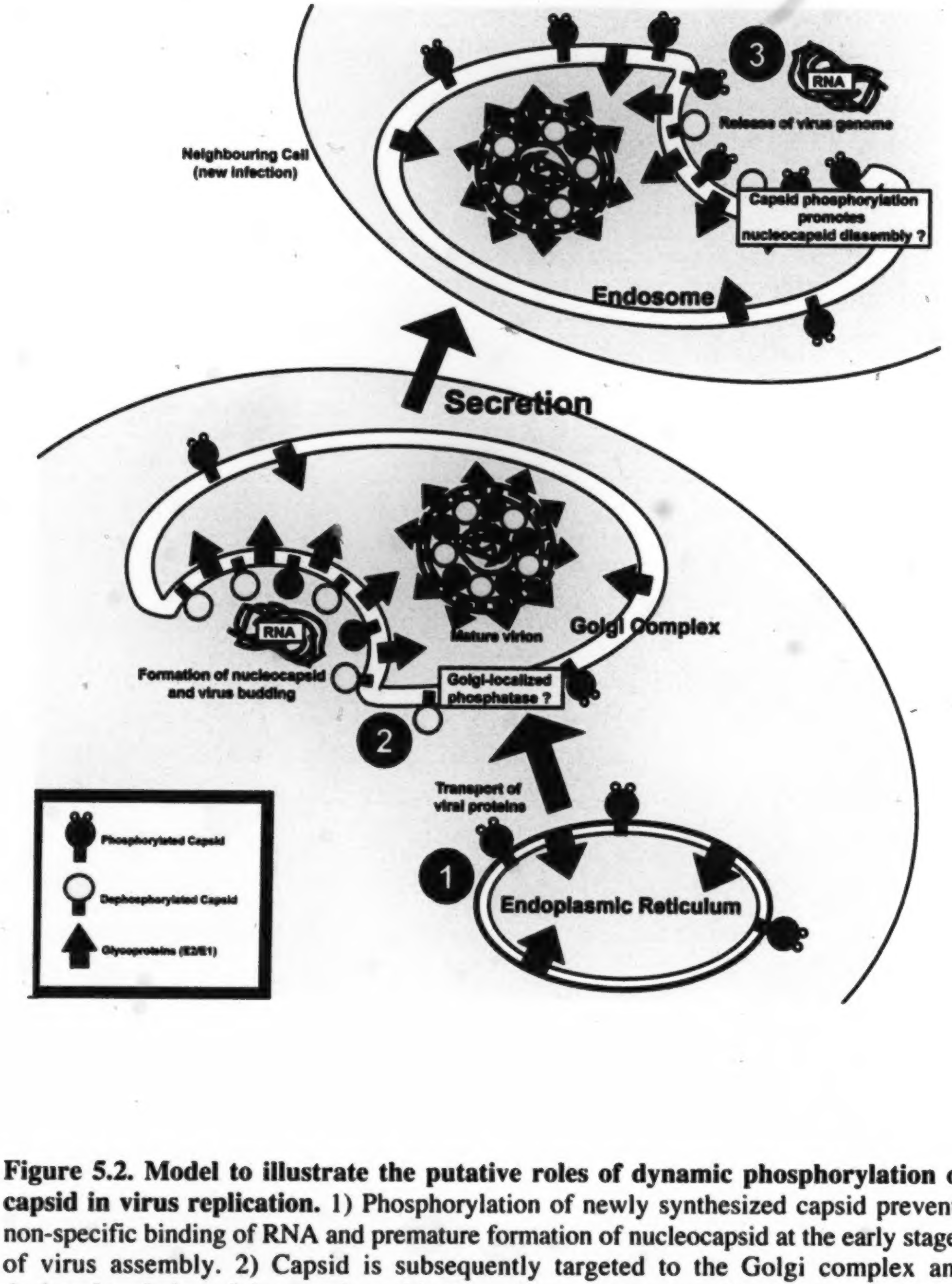
5.3.1 Identification of RV capsid phosphorylation sites

I have shown that the RV capsid is multiphosphorylated with the majority of phosphorylated residues concentrated within the RNA binding site. Based on analyses with three algorithms, capsid was determined to have up to 19 different potentially phosphorylated serine/threonine residues, seven of which are clustered within the RNA binding site (Figure 4.1). Using alanine-scanning mutagenesis, I have determined that the majority of phosphorylated residues are indeed located within the RNA binding site. Of these residues, serines 46, 48, 52 and threonine 47 are likely to be the major phosphorylated amino acid residues. Serine 46 is the critical amino acid residue with respect to regulating phosphorylation of capsid. Phosphorylation of this residue is necessary for progressive phosphorylation of other amino acid residues within the RNA binding site. Since acidic residues (aspartate or glutamate) at position 46 can mimic the effect of phosphorylated serine in terms of allowing downstream phosphorylation, it is likely that the negative charge at this location triggers an as yet unidentified kinase to phosphorylate other amino acid residues.

If capsid is multiphosphorylated, it is conceivable that there are various pools of differentially phosphorylated capsids. Both wild type and hypophosphorylated capsids (e.g. CapS46A) appear to resolve similarly as a doublet using SDS-PAGE, suggesting the doublet is not due to differential phosphorylation of capsid proteins. Using two dimensional gel electrophoresis, capsid can be separated into two isoelectric species of pH 8.8 and pH 9.5 (Waxham and Wolinsky, 1985). However, I was unable to consistently resolve capsid into multiple species using the same methods. It will be of interest to understand if the two isoelectric species of capsid identified by Waxham *et al.* correspond to either phosphorylated/nonphosphorylated capsids or different pools of differentially phosphorylated capsids.

5.3.2 The function of capsid phosphorylation in virus replication

Substitution of serine 46 with alanine blocks phosphorylation of multiple residues in the RNA binding site, resulting in hypophosphorylated capsids. Because of this feature, I used the mutant CapS46A as a "non-phosphorylatable capsid" to study the role of phosphorylation in virus replication. As capsid is heavily phosphorylated prior to, but not following, virus assembly, I propose that phosphorylation of capsid negatively regulates its interaction with viral RNA early in the assembly pathway. At a later step, dephosphorylation of capsid is required before RNA binding and efficient nucleocapsid assembly can occur (Figure 5.2). In agreement with this model, the level of phosphorylation in virion associated capsids is reduced compared to cell associated capsids (Figure 4.6). This is consistent with a scenario whereby a fraction of capsid is dephosphorylated prior to the formation of nucleocapsids.



Neighbouring Cell
(new infection)
3
RNA
Release of virus genome
Capsid phosphorylation promotes nucleocapsid disassembly ?
Endosome
Secretion
RNA
Mature virion
Golgi Complex
Formation of nucleocapsid and virus budding
Golgi-localized phosphatase ?
2
Transport of viral proteins
1
Endoplasmic Reticulum
Phosphorylated Capsid
Dephosphorylated Capsid
Glycoproteins (E2/E1)

Figure 5.2. Model to illustrate the putative roles of dynamic phosphorylation of capsid in virus replication. 1) Phosphorylation of newly synthesized capsid prevents non-specific binding of RNA and premature formation of nucleocapsid at the early stages of virus assembly. 2) Capsid is subsequently targeted to the Golgi complex and dephosphorylation of the protein at this stage allows interaction with the genomic RNA, formation of the nucleocapsid and subsequent virus budding. 3) Timely re-phosphorylation of capsid before or during virus entry promotes the disassembly of nucleocapsid.

Why is it important to regulate the RNA-binding activity of capsid? First, it is tempting to speculate that capsids that are free of RNA may be transported from the site of synthesis (ER) to the budding site (Golgi) easier than fully formed nucleocapsids. In keeping with this hypothesis, our lab previously showed that capsid is still phosphorylated normally under conditions where its transport to the virus assembly site is blocked (Garbutt et al., 1999). Unlike alphavirus capsids, about 50% of RV capsid remains membrane-bound (this study and (Suomalainen et al., 1990)) and thus cannot simply move by diffusion from the site of synthesis to the budding site. Based on the results of this study, capsid is likely transported to the Golgi complex via vesicular transport. If this is the case, premature assembly of bulky nucleocapsid structures on the ER surface could inhibit the recruitment of coat proteins needed for formation of transport vesicles that ferry capsid to the Golgi region. Secondly, phosphorylation of capsid might serve to prevent non-specific RNA binding. In particular during the early phase of replication, the ratio of virus genomic RNA to cellular RNAs is presumed to be very low. Since non-phosphorylated capsid is a very basic protein, it may have a propensity to bind non-specifically to nucleic acids, a situation that could interfere with binding of virus genomic RNA at a later stage. The negative charge on the phosphorylated residues in the RNA-binding region may prevent non-specific low affinity electrostatic interaction with RNA. Alternatively, phosphorylation of capsid may alter its confirmation in a manner that inhibits RNA binding. For example, phosphorylation regulates the conformation of the duck hepatitis virus capsid protein (Yu and Summers, 1994b). It is conceivable that phosphorylation of RV capsid may regulate the exposure of the highly charged RNA binding site.

Characterization of recombinant virus encoding the mutation of Serine 46 to alanine (S46A virus) revealed that this strain has a defect at, or prior to, the step of uncoating. This observation is in agreement with my proposed model that capsid phosphorylation is dynamic, which could facilitate both the efficient binding of viral RNA and release of RNA at the appropriate points in the replication cycle. Initially, I predicted that phosphorylation of capsid by a cellular kinase could destabilize the nucleocapsid to release the genomic RNA during virus entry (Figure 5.2). This process would presumably afford easier access of ribosomes to the genomic RNA allowing translation of the non-structural proteins to occur with greater efficiency. For example, encapsidated RNA of potato virus X is non-translatable in vitro, but can be rendered translatable after phosphorylation of the coat protein (Atabekov et al., 2001). However, I was unable to detect a change in the level of capsid phosphorylation during virus uncoating (Figure 4.10). This seems to argue against the possibility that phosphorylation of capsid promotes the disassembly of nucleocapsid.

How does S46A mutant virus delay the accumulation of viral proteins/RNA? One of the simplest explanations is that the intrinsic high affinity of hypophosphorylated capsid (S46A) for RNA delays nucleocapsid disassembly. In secreted wild type virions, capsid retains approximately 40% of the phosphorylation level of intracellular capsids, indicating that capsid is not completely dephosphorylated (Figure 4.6). It is possible that the pool of phosphorylated capsids serve to "destabilize" the nucleocapsid in order to facilitate releasing of genome. In contrast, the nucleocapsid of S46A mutant virus would be expected to contain only non-phosphorylated capsids that have high RNA affinities. During virus disassembly, it is conceivable that capsid proteins are normally in a metastable conformation that accommodates the release of the virus genome. In the wild type situation, the presence of phosphorylated capsid proteins within the nucleocapsid at this stage, may be required for efficient release of the virus genome during entry. In contrast, the strong capsid-RNA interaction of the nucleocapsids in S46A virus may slow down the process of nucleocapsid disassembly resulting in delayed synthesis of both viral RNA and proteins. I hypothesize that the balance of phosphorylated/non-phosphorylated capsids within the nucleocapsid is essential to maintain the nucleocapsid integrity without compromising the efficiency of virus uncoating.

Failure to detect capsid rephosphorylation during virus entry may reflect the possibility that phosphorylation of capsid occurs at an earlier stage. For example, in other viruses such as hepatitis B virus and human immunodeficiency virus (HIV), protein kinases are known to be packaged within virions (Cartier et al., 1997; Cartier et al., 2003; Daub et al., 2002). In the latter case, the activity of the virion-associated is required for HIV infectivity (Cartier et al., 2003; Hemonnot et al., 2004). I examined the phosphorylation of capsid in secreted virions (Figure 4.6). The relatively low level of capsid phosphorylation signal in virions could result from rephosphorylation of capsids after completion of virus assembly by a virion-associated kinase. This is clearly speculative as it is currently unknown if any kinases are associated with rubella virions. Proteomic approaches to identify host-associated protein(s) within rubella virions could address this possibility. The presence of a host cell kinase within virions would support my model that timely phosphorylation of capsid is essential for mediating genomic RNA release (Figure 5.2).

5.3.3 Host cell regulation of capsid phosphorylation

Identifying the kinases and phosphatases that are responsible for regulating capsid phosphorylation is critical for understanding where and when these processes occur. The kinase(s) responsible for phosphorylation of capsid is presently unknown. However, sequence analyses using algorithms such as Scansite or NetPhos indicate that Serine 46 is a potential substrate for cAMP-dependent protein kinase (PKA), protein kinase B (PKB/Akt), the 70 kDa ribosomal protein S6 kinase (p70S6K) or calmodulin-dependent kinase. Due to the fact that capsid is multiphosphorylated, it is very likely that there are multiple kinases that phosphorylate different amino acid residues in capsid. For example, according to NetPhos, threonine 47 and serines 48, 52 are predicted to be phosphorylated by casein kinase II (CKII). Since phosphorylation of serine 46 had a dramatic effect in regulating progressive downstream phosphorylation, I initially focused on how phosphorylation of this amino acid residue is regulated.

The above mentioned kinases all have broad specificity and are involved in various aspects of cellular signaling. Their involvement in signaling processes necessitates that their activities or access to substrates are regulated. Restricting the localizations of protein kinases is one way that eukaryotic cells modulate the activity of these enzymes (Hubbard and Cohen, 1993; Mochly-Rosen, 1995; Pawson and Scott, 1997). Differential localization of a given kinase can be mediated by binding to a variety of anchoring proteins each with different targeting signals (Huang et al., 1999). Presumably, phosphorylation of nascent capsid is a process that occurs on the surface of the ER. In this respect, both PKA and calmodulin-dependent kinases have been localized to the ER (Kosmopoulou et al., 1994). However, there is no report that documents the association of active PKB/Akt with the ER. Rather, activated forms of these kinases are known to translocate to the plasma membrane, after which they detach from this site and move to the nucleus (Andjelkovic et al., 1997). In this regard, p70S6K is very similar to Akt/PKB in that it is a cytosolic protein that can translocate to the nucleus (Valovka et al., 2003) or be recruited to the plasma membrane for participation in cellular signaling events (Buchsbaum et al., 2003). Preliminary data suggest that PKA may be important for phosphorylation of capsid since phosphorylation of capsid is inhibited by the PKA specific inhibitor H-89 (Figure A.1). However, the role of PKA in capsid phosphorylation needs to be further examined because the doses required for inhibition in my experiments were relatively high (μM range) (Aridor and Balch, 2000).

Formation of RV nucleocapsids is generally coordinated with virus budding and pre-formed nucleocapsids are rarely observed in RV-infected cells. In the context of my model (Figure 5.2), this would require that capsid dephosphorylation occurs at the virus budding site and therefore that nucleocapsid assembly may be regulated by a Golgi localized phosphatase. Thus, identification of the enzyme that dephosphorylates capsid would provide insight into the control of virus assembly. In eukaryotes, the major groups of serine/threonine protein phosphatases display slightly different, but overlapping preferences for substrates (reviewed in (Cohen, 1997)). For example, phosphorylated serines or threonines located downstream of multiple basic residues are preferred substrates for PP1 and PP2A (Agostinis et al., 1990; Donella-Deana et al., 1994). As serine 46 of capsid is downstream from a group of arginine residues, it is a potential substrate for both of these enzymes. However, PP1A is the most likely candidate since this enzyme, but not PP2A, efficiently dephosphorylated capsid *in vitro* (Figure 4.7). In addition, PP1 isoforms are required for membrane trafficking steps near the RV budding site (Peters et al., 1999) and thus are in a position to dephosphorylate RV capsid at the appropriate time and place. However, further studies are clearly required to verify this model.

Capsid phosphorylation may not only be important for virus replication, but for virus host interactions as well. For example, RV strains with hypophosphorylated capsids are less cytopathic in certain cell types (Figure 4.3B). In this respect, I hypothesize that the phosphorylation state of capsid may also affect its interactions with host proteins. My results support the idea that interaction of capsid with host cell proteins is phosphorylation dependent (Figure A.2). Further characterization of these host proteins is required to understand the significance of these interactions. There are several possible mechanisms to regulate capsid-host interaction via phosphorylation. One possibility is that phosphorylation of capsid alters the conformation of the protein, therefore allowing interaction with a different subset of host proteins. For example, the endo-domain of the Sindbis virus E2 protein undergoes a conformational change after phosphorylation, which allows interaction with the nucleocapsid core (Liu et al., 1996a). In addition, phosphorylation of avian retrovirus nucleocapsid changes the conformation of the protein in order to expose the charged region of the protein (Fu et al., 1985). Alternatively, phosphorylation may be important for regulating the subcellular localization of the capsid such that it interacts with a different set of host proteins. Indeed, phosphorylation of capsids of both hepatitis B virus and hepatitis C virus is essential for shuttling these proteins to the nucleus (Kann et al., 1999; Lu and Ou, 2002; Rabe et al., 2003).

5.3.4 Mechanisms regulating capsid phosphorylation

My data suggest that phosphorylation of capsid is the molecular switch that regulates capsid-RNA interactions (Figure 4.5). Subsequent experiments using recombinant capsids in which serine 46 was replaced with acidic amino acid residues revealed that the mechanisms regulating capsid phosphorylation are more complex than originally thought. For instance, the capsid-RNA interaction does not appear to be solely dependent on the phosphorylation of Serine 46 *per se*. Rather, the presence of phosphoserine at position 46 seems to be important to induce downstream phosphorylation of other amino acid residues. Moreover, the negative charge of acidic residues at position 46 can mimic the effect of phosphoserine such that progressive downstream phosphorylation occurs. However, neither aspartate nor glutamate can mimic the effect of phosphorylation in blocking the capsid-RNA interaction. This is exemplified by recombinant constructs such as CapP6D and CapP3E, in which most of the potentially phosphorylated amino acid residues were replaced with either aspartate or glutamate residues, exhibit increased RNA binding activity, a situation similar to other hypophosphorylated capsids (Table 4.1). To this point, I was unable to construct a recombinant capsid mutant that mimics faithfully all aspects of constitutively phosphorylated capsid.

Although capsid constructs that have serine 46 replaced with either aspartate or glutamate both allow progressive phosphorylation, these proteins (CapS46D and CapS46E) exhibit different RNA binding activities. Specifically, CapS46E exhibits a higher affinity for RNA than CapS46D (Table 4.1). Relative to the size of a phosphate group, replacement with glutamate is considered to be a less conservative mutation than replacement with aspartate. Whether or not this can account for the discrepancy between CapS46D and CapS46E requires further investigation. Moreover, it is unknown if different residues are phosphorylated in CapS46D and CapS46E.

Further analysis of the capsid mutants revealed that not all the phosphorylated amino acid residues contributed equally to the regulation of the capsid-RNA interaction. Based largely on the fact that relatively high RNA binding activities were associated with CapA3, CapA4-2 and CapP3D (Table 4.1), I suggest that threonine 47 is the most important residue for regulating the capsid-RNA interaction. CapT47A, despite being heavily phosphorylated, demonstrated high RNA binding activity. Therefore, replacing threonine 47 with alanine influences the RNA binding activity of capsid. However, threonine 47 might not be the only amino acid residue that regulates the capsid-RNA interaction since phosphatase treatment of CapT47E restored the RNA binding activity to the level of CapT47A (Figure 4.12B). This suggests that other unidentified phosphorylated residue(s) may be essential to regulate the capsid-RNA interaction. If this is true, it is likely that the unidentified residue(s) is not phosphorylated in the CapT47A mutant because this construct binds RNA much more efficiently than CapT47E prior to treatment with phosphatase (Figure 4.12B). These data are also consistent with the possibility that an acidic residue or phosphorylation at position 47 induces downstream phosphorylation of other amino acid residues, a situation similar to the effect of acidic residues at position 46. However, based on the observation that CapT47A and CapT47E incorporate similar levels of radioactive-phosphate (Figure 4.11A), it is unlikely that CapT47E contains additional phosphorylated amino acid residues. More work is certainly required to define the role of threonine 47 in regulating the capsid-RNA interaction.

I have so far been unable to identify a key phosphorylated residue that directly regulates the capsid-RNA interaction alone. It is possible that regulation of the capsid-RNA interaction is the result of the concerted action of multiple amino acid residues. Alternatively, the role of capsid phosphorylation may be to modulate the conformation of the RNA binding site to prevent RNA binding. It is interesting to note that majority of phosphorylated residues in capsid (serines 46, 48, 52 and threonine 47) are located between two stretches of arginines residues. These arginine residues are important for RV replication (Beatch, 2004). In retroviruses, it has been shown that basic amino acid residues are important for RNA packaging (Lee et al., 2003). Further experiments are needed to determine if phosphorylation and the arginine residues of RV regulate the capsid-RNA interaction in concert. A similar model has been proposed for the regulation of p12, a major nucleocapsid protein of avian retroviruses. Phosphorylation of this protein has been shown to regulate its RNA binding activity (Leis et al., 1984). A subsequent study revealed that phosphorylation of p12 regulates the conformation of the protein to expose lysine residues near the phosphorylated serine residue (Fu et al., 1985). To address this possibility in RV, identification and characterization of revertants that overcome the replication defect of the S46A virus may provide some insight. For example, multiple revertant clones of moloney murine leukemia virus encoding the phosphorylation mutant of p12 have incorporated mutations that introduce new arginine residues around the original phosphoserine residue (Yueh and Goff, 2003). If the arginine residues of RV capsid are essential for the RNA binding activity, some revertants would be expected to have a higher number of arginine residues near the phosphorylated serine/threonine residues.

In retrospect, site-directed mutagenesis may not be the ideal method to identify specific phospho-serine/threonine residues since phosphorylation of one residue may be dependent upon the phosphorylation state of another. Identifying how each of the site-directed mutations affects phosphorylation of capsid using mass spectrometry and subsequently determining the RNA binding activity of these constructs would be a more comprehensive approach. This may provide a better understanding of how each phosphorylated residue contributes to the overall RNA binding activity of the protein. By the same token, identification of specific phosphorylated residues in wild type capsid may help to understand if the pattern of phosphorylated residues changes during the life cycle of RV. Given that capsid is multiphosphorylated, multiple kinases that localize to different compartments in the cell may be involved. Finally, it is possible that the phosphorylation pattern of capsid reflects several pools of capsid at different subcellular locations. Numerous attempts were made to identify phosphorylated residues in capsid using mass-spectrometry, however, technical problems were encountered.

5.3.5 Phosphorylation of capsids in related RNA viruses

It was important to determine whether capsid phosphorylation is a general mechanism that regulates nucleocapsid assembly for other positive strand RNA viruses. To this end, I tested whether two capsid proteins of related positive strand RNA viruses become stably phosphorylated: Sindbis virus capsid (*Togaviridae*) and West Nile virus core (*Flaviviridae*). Unfortunately, I was unable to find any evidence that these two capsid proteins are phosphorylated (Figure A.3). The most direct interpretation of these results is that these viruses have evolved other mechanisms to regulate capsid-RNA interactions.

The failure to detect the phosphorylation of the Sindbis capsid protein contradicts a previous study, which indicated the Sindbis capsid is phosphorylated (Waite et al., 1974). One of the reasons to account for this discrepancy could be that their experiments were done in chicken cells, whereas mine were carried out in COS (monkey kidney) or Vero (African green monkey kidney) cells. Furthermore, detection of Sindbis virus protein phosphorylation may require the employment of additional protein phosphatase inhibitors, such as okadaic acid (Liu and Brown, 1993). If Sindbis virus capsid utilizes a similar mechanism as the RV capsid to regulate formation of nucleocapsids (i.e. dephosphorylation of capsid is required to increase the RNA binding and the nucleocapsid formation), most of the intracellular Sindbis virus capsids would presumably be dephosphorylated. This is because formation of alphavirus nucleocapsid is readily observed in the cytoplasm of infected cells (Froshauer et al., 1988). Accordingly, the inability to detect Sindbis virus capsid phosphorylation may reflect this fact. Regarding the West Nile core protein, there are only very limited details known about the nucleocapsid assembly pathway. However, for other flaviviruses, such as hepatitis C virus, the core protein is reported to be phosphorylated but it is not known if this regulates RNA binding (Lu and Ou, 2002). Nonetheless, there are other examples where viruses use phosphorylation to regulate the RNA binding activities of viral proteins (Cuesta et al., 2000; Gazina et al., 2000; Ivanov et al., 2001; Rabe et al., 2003; Yueh and Goff, 2003). Clearly, more work is needed in both cases of Sindbis virus and West Nile virus capsid/core proteins.

5.4 Model for RV assembly

In this study, the functional roles of two capsid regions during virus replication were characterized. These results have greatly increased our understanding regarding the assembly of RV nucleocapsids. Together, the E2 SP and phosphorylation of amino acid residues within the RNA binding site of capsid could provide the spatial and temporal control required to coordinate the processes of nucleocapsid formation and virus budding. Nucleocapsid formation is a regulated event that coincides with virus budding at the Golgi complex. Since viral proteins are synthesized at the ER, the virus must employ a mechanism to ensure that coordinated assembly takes place at the Golgi complex. From the results of this study, I propose that the function of capsid phosphorylation at the early stage of infection is to prevent capsid binding to genomic RNA, thereby delaying formation of the nucleocapsid. The role of the E2 SP at this point is to provide a means to interact with the virus glycoproteins, probably E2, thereby ensuring proper targeting to the Golgi complex.

Once capsid reaches the Golgi complex, a Golgi localized phosphatase, possibly PP1A, dephosphorylates capsid, which allows interaction with the genomic RNA. The delay of capsid binding to genomic RNA until reaching to the Golgi complex, not only serves to ensure efficient transport of structural proteins, it could also function to allow a time window for genome amplification. The interaction of capsid and genomic RNA at this stage presumably provides the driving force that induces formation of nucleocapsids.

The RV capsid is involved in two major driving forces that regulate virion assembly: 1) The capsid-RNA interaction (formation of the nucleocapsid) and 2) the capsid-glycoprotein interaction (virus budding). In alphaviruses, these events are separated spatially and temporally and it is the interaction of preformed nucleocapsids and the glycoproteins (capsid-glycoprotein) that drives virus budding at the plasma membrane (Zhao et al., 1994). In rhabdoviruses, virus budding occurs by a "push and pull" mechanism where nucleocapsid (bending of the membrane from the inside) and membrane proteins (bending of the membrane from the outside) act in concert to drive virus budding (Mebatsion et al., 1996). The assembly pathway of RV is expected to more closely resemble that of rhabdoviruses. Dephosphorylation of capsid could allow for interaction with the genomic RNA to drive the formation of nucleocapsids. At the same time, the lateral interactions between the E2 SP at the carboxyl-terminus of capsid and other membrane spanning domains of the glycoproteins could bend the membrane in order to drive virus budding. In this model, the interactions of capsid-RNA and of capsid-glycoprotein act coordinately to induce virus budding. This suggests that RNA might act as an essential structural element to initiate virus budding.

If RNA is essential for virus assembly, how does expression of RV structural proteins result in formation of RLPs? For retroviruses at least, virus-like particles can form after binding cellular RNAs (e.g. ribosomal RNA) in the absence of virus genome (Muriaux et al., 2001). Determining if RLPs contain cellular RNAs would shed light on the role of RNA during RV assembly. If RLPs are devoid of RNA, it may indicate that the driving force for virus budding is initiated from the capsid-glycoprotein interaction. Indeed, expression of some viral glycoproteins is sufficient to induce budding and formation of liposomes (Justice et al., 1995; Li et al., 1993). It is possible that accumulation of RV glycoproteins at the Golgi complex is sufficient to induce bending of the membrane to initiate, but not complete, the budding process.

The results from experiments in this thesis do not distinguish whether or not the E2 SP is needed for virus budding. It would be of interest to replace the E2 SP on capsid with a Golgi localization signal (e.g. the E2 TM) in order to test whether or not the E2 SP has dual functions in targeting capsid to the Golgi complex and in driving virus budding via interaction with the glycoproteins.

5.5 Conclusion

This work provides a mechanistic understanding of how RV assembly is coordinated. Moreover, it further highlights the differences between the assembly pathways of RV and the structural similar alphaviruses. With the newly identified functions of capsid in virus replication, understanding the nature of the capsid-RNA interaction could help to understand the control of virus replication.

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Appendix

A.1 Effect of kinase-specific inhibitors on capsid phosphorylation

Since phosphorylation at serine 46 is required for normal phosphorylation of RV capsid, it is essential to understand how phosphorylation of this site is regulated. Sequence analysis using algorithms such as Scansite and NetPhos indicate that serine 46 is a potential substrate for PKA, protein kinase B (PKB/Akt), p70S6K or calmodulin-dependent kinase. Based on this information, several kinase specific inhibitors (H-89, Wortmannin, Rapamycin and Ro-31-8220) were selected in an attempt to identify the kinase(s) responsible for capsid phosphorylation (Figure A.1). H-89 is a PKA-specific inhibitor (Lu and Ou, 2002; Wadhwa et al., 2002); Wortmannin is a phosphatidylinositol 3-kinase (PI3K) inhibitor which blocks the subsequent activation of PKB/Akt (Bandyopadhyay et al., 2002; Shelly and Herrera, 2002); Rapamycin is a p70S6K inhibitor (Guizzetti and Costa, 2002); Ro-31-8220 is a general inhibitor of PKCα, β and γ isoforms (Lu and Ou, 2002). The PKC inhibitor was chosen since capsid binds to the host protein p32 (Beatch and Hobman, 2000), which has been shown to regulate the activities of RNA binding proteins and the activities of all classes of PKC (Petersen-Mahrt et al., 1999; Robles-Flores et al., 2002). Of the inhibitors tested, H-89 significantly inhibited the phosphorylation of capsid at 30 μM. Although the treatment of Wortmannin at 200 nM appeared to partially inhibit capsid phosphorylation, subsequent experiments confirmed that Wortmannin does not consistently affect capsid phosphorylation even at higher doses than 200 nM. Despite using a high dose of H-89, I never observed a complete inhibition of capsid phosphorylation. In addition, these results are complicated by the fact that, at higher doses of H-89 (>30 μM), expression or stability of capsid is decreased in

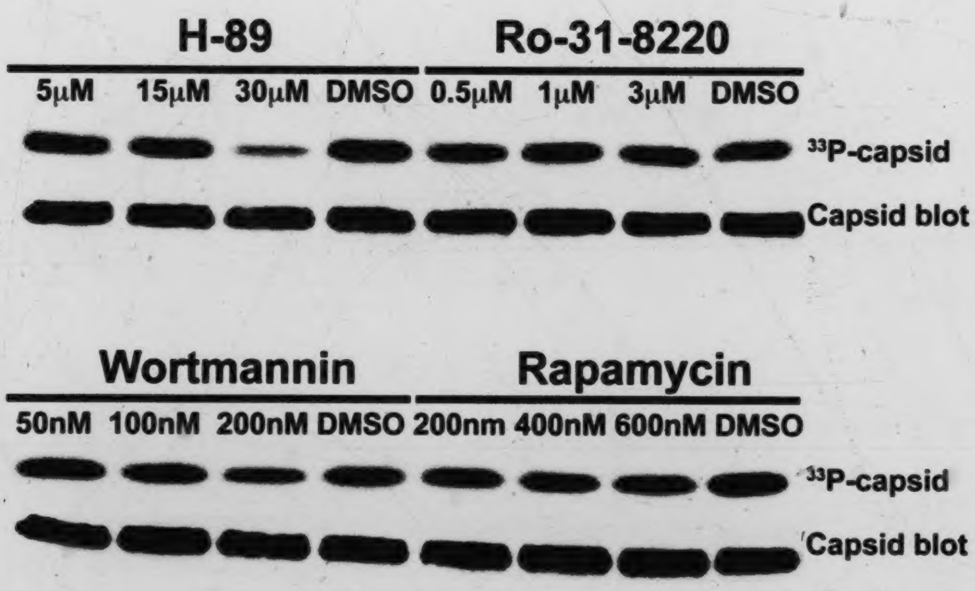

H-89
Ro-31-8220
5μM
15μM
30μM
DMSO
0.5μM
1μM
3μM
DMSO
33P-capsid
Capsid blot
Wortmannin
Rapamycin
50nM
100nM
200nM
DMSO
200nm
400nM
600nM
DMSO
33P-capsid
Capsid blot

Figure A.1. The effect of various kinase inhibitors on capsid phosphorylation. Plasmids encoding wild type capsid were transiently expressed in COS cells. Twenty-four hours post-transfection, cells were incubated for 30 minutes in phosphate-free media containing specified kinase inhibitors (H-89, Ro-31-8220, Wortmannin or Rapamycin). Cells were then labeled (in the presence of kinase inhibitors) with media containing 100 μl [^{33}P]-orthophosphate for 12-16 hours. After labeling, cells were lysed in RIPA buffer and capsids were immunoprecipitated. Samples were subjected to SDS-PAGE and processed for fluorography. Top panels represent results of radiography and bottom panels represent results of western blotting.

transiently or stably transfected cells expressing capsid. The reason for this phenomenon is not known. However, it is not likely due to a higher turnover rate of "unphosphorylated" capsid because the stability of wild type capsid and hypophosphorylated capsid, CapS46A, are similar. Other PKA-related inhibitors should be tested in order to further confirm the effect of PKA on capsid phosphorylation.

A.2 Host cell interactions with capsid phosphorylation mutants

When cells were infected with virus strains encoding hypophosphorylated capsids, it was observed that they exhibited less cytopathic effects (Figure 4.3B). This phenomenon could be due to the lower replication rates of the mutant viruses. Alternatively, phosphorylation of capsid may regulate capsid-host interactions that affect cell viability (i.e., differentially phosphorylated capsids may interact with different subsets of host proteins). To test the latter scenario, GST was fused to the amino terminus of either the wild type capsid or recombinant capsids encoding the alanine mutations (S45A, S46A, T47A). Plasmids encoding the capsid or the recombinant capsids were transiently transfected into COS cells. Forty-eight hours post-transfection, protein complexes that interact with GST-capsid fusion proteins were purified using glutathione-agarose beads, subjected to SDS-PAGE and visualized by silver staining. As a negative control, plasmids encoding GST alone were used. Several proteins were found that specifically associated with the hypophosphorylated capsid (CapS46A) (Figure A.2). On the other hand, at least one unique protein was associated only with normally phosphorylated capsids (WT, CapS45A, CapT47A) (Figure A.2). The identities of these proteins remain to be determined. However, since all these protein bands migrated below

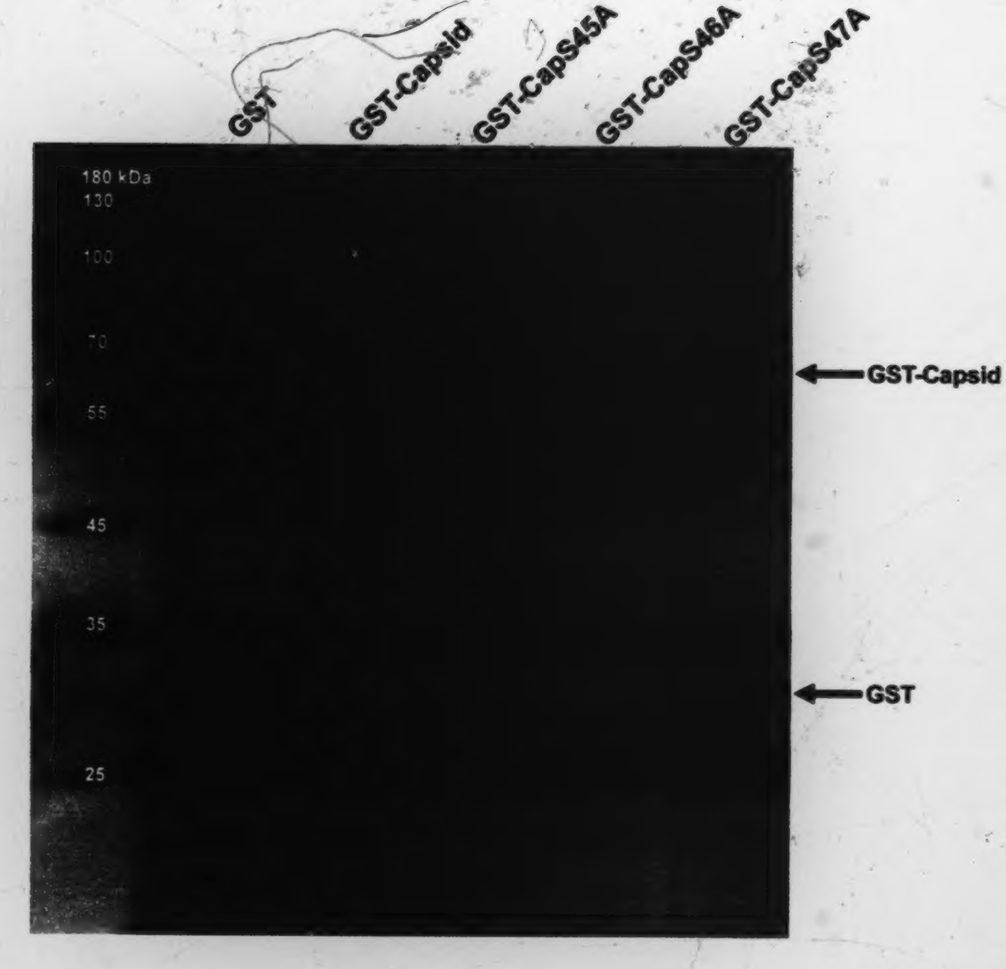

GST
GST-Capsid
GST-CapS45A
GST-CapS46A
GST-CapS47A
180 kDa
130
100
70
55
45
35
25
GST-Capsid
GST

Figure A.2. Interactions between wild type and mutant capsids with host proteins. COS cells (in 60mm dish) were transiently transfected with plasmids encoding GST, GST-Capsid, GST-CapS45A, GST-CapS46A or GST-T47A. Forty-eight hours post-transfection, cells were lysed with 1% NP40 lysis buffer and processed for GST-pulldown using glutathione-agarose beads. The protein complexes bound to glutathione agarose-beads were washed and eluted by boiling in 2 x SDS protein gel loading buffer. Samples were then subjected to SDS-PAGE and silver-staining. (*) Proteins that are specifically pulled-down by GST-capsid and other phosphorylated capsids (CapS45A and CapS47A); (->) Proteins that are specifically pulled-down by hypophosphorylated capsid, CapS46A.

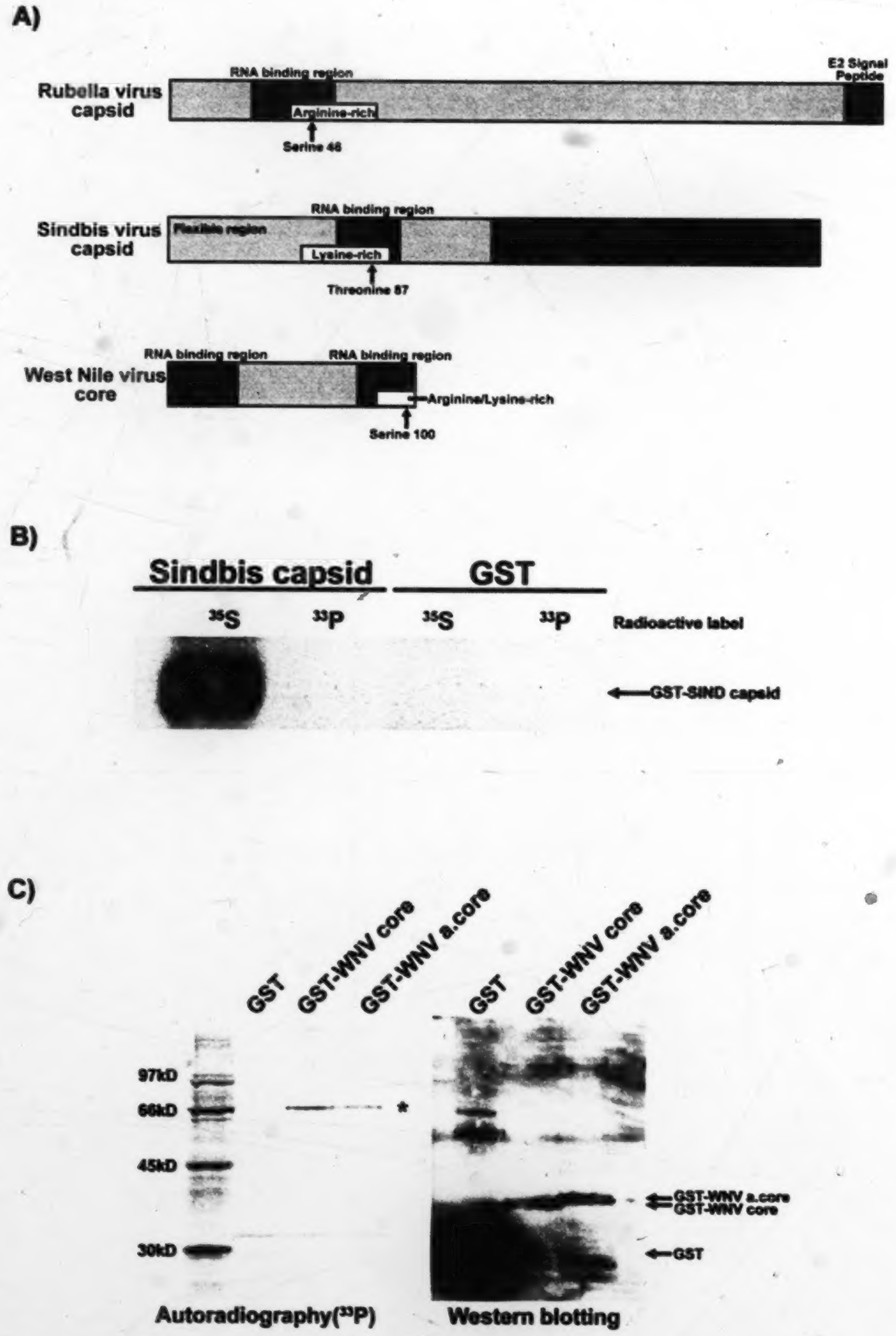
the GST-capsid fusion protein bands, it cannot be ruled out that they are degradation products of the fusion proteins.

A.3 Capsids of two related RNA viruses are not stably phosphorylated

I have demonstrated that phosphorylation is a mechanism by which RV capsid can regulate nucleocapsid formation. The bigger question, of course, is whether or not this mechanism is shared by other related viruses. Interestingly, there are potential phosphorylation sites within the RNA binding regions of capsid proteins from related positive strand RNA viruses such as the Sindbis virus (*Togaviridae*) and the West Nile virus (*Flaviviridae*) (Figure A.3A). Note that the capsid protein of the latter virus is also referred to as core protein. To determine if Sindbis and West Nile virus capsid proteins were phosphorylated, GST-capsid fusion proteins were created. COS cells, transiently expressing GST-Sindbis capsid or GST-West Nile core, were labeled with [^{33}P]-orthophosphate as described for RV capsid. Under these conditions, these capsid proteins were not labeled with radioactive phosphate (Figure A.3 B and C), whereas RV capsid is heavily labeled. The expression of these proteins was confirmed by [^{35}S] metabolic labeling in the case of Sindbis virus capsid (Figure A.3B) and western blotting using GST-antibodies in the case of West Nile virus core (Figure A.3C). The inability to show that the Sindbis virus and West Nile virus capsid/core proteins are phosphorylated suggests that these viruses may have evolved different mechanisms for regulating capsid-RNA interactions and subsequent nucleocapsid formation.

Similar to the RV capsid, a pool of the West Nile core protein is membrane associated. Membrane association is conferred by a short hydrophobic domain at the carboxyl end, which is subsequently removed by a virus-encoded protease late in

Figure A.3. Sindbis virus capsid and West Nile virus core proteins are not stably phosphorylated. A) Schematic representation of RV capsid, Sindbis virus capsid and West Nile virus core proteins. The RNA binding domains are represented by blue boxes and regions of high positive charge density are marked by white boxes. The RNA binding regions of each capsid/core were predicted by the Scansite algorithm to have either potentially phosphorylated serine or threonine residue (arrows). B) Twenty-four hours post-transfection, COS cells transiently expressing GST-Sindbis capsid fusion proteins or GST alone were labeled either with [^{35}S]-Methionine/Cysteine or with [^{33}P]-orthophosphate for 16 hours. The fusion proteins were purified on glutathione-sepharose beads. Eluates were subjected to SDS-PAGE and fluorography. C) Similar to the experiment described in B, COS cells transiently expressing GST protein, GST-West Nile core (GST-WNV core) or GST-West Nile anchored core (GST-WNV a. core) were labeled with [^{33}P]-orthophosphate for 16 hours. Samples were subjected to purification using glutathione-sepharose beads, followed by SDS-PAGE and fluorography. An unidentified phosphoprotein with an apparaent molecular weight of 70 kDa (*) co-purifies with the GST-WNV core and the GST-WNV a. core. Expression of the GST-fusion proteins was confirmed by probing the same membranes with anti-GST antibodies followed by ECL detection (right panel).


A)
Rubella virus capsid
RNA binding region
E2 Signal Peptide
Arginine-rich
Serine 46
Sindbis virus capsid
RNA binding region
Flexible region
Lysine-rich
Threonine 87
West Nile virus core
RNA binding region
RNA binding region
Arginine/Lysine-rich
Serine 100
B)
Sindbis capsid
GST
35S
33P
35S
33P
Radioactive label
GST-SIND capsid
C)
GST
GST-WNV core
GST-WNV a.core
GST
GST-WNV core
GST-WNV a.core
97kD
66kD
45kD
30kD
*
GST-WNV a.core
GST-WNV core
GST
Autoradiography(33P)
Western blotting

infection (Brinton, 2002). The membrane bound form of core is known as anchored core. Experiments were preformed to test if the membrane anchor of the West Nile virus core was required for phosphorylation of the protein. However, I was unable to detect phosphorylation of the anchored core (Figure A.3C). Interestingly, both forms of West Nile core proteins (core and anchored core) interacted with an unidentified 70 kDa phosphoprotein (Figure A.3).